THE ROLE AND MECHANISM OF IMMUNOMODULATION BY INFLUENZA VIRUS AND ITS COMPONENTS IN THE PREDISPOSITION TO BACTERIAL DISEASE DURING INFLUENZA INFECTION

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DECLARATION

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TABLE OF CONTENTS

ABBREVIATIONS

Abs	Absorbance
AO	Acridine orange
APC	Antigen presenting cell
BMDC	Bone marrow-derived immature DCs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
cRPMI	Complete Roswell Park Memorial Institute
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EtBr	Ethidium bromide
ETDA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
gDNA	Genomic deoxyribonucleic acid
GM-CSF	Granulocyte-macrophage colony stimulating factor
HA	Haemagglutinin
HBSS	Hanks balanced salt solution
HKSP	Heat killed *Streptococcus pneumoniae*
HLA	Human leucocyte antigen
HPIV3	Human parainfluenza virus type 3
HRP	Streptavidin horseradish peroxidase
IAV	Influenza A Virus
IBV	Influenza B Virus
IFN	Interferon
IFN-β	Interferon-β
IFNAR	IFNα/β receptor
IL	Interleukin
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor

LAIV	Live attenuated quandrivalent influenza vaccine
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
M	Matrix protein
mAb	Monoclonal antibody
MAL	MyD88-adaptor-like
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
mRNA	Messenger ribonucleic acid
MyD88	MyD88 innate immune signal transduction adaptor
NA	Neuraminidase
NI	Neuraminidase inhibitors
NF-κB	Nuclear factor-κB
NK	Natural Killer
NP	Nucleoprotein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
NEP	Nuclear export protein
OD	Optical density
PA	Acidic polymerase
PAMP	Pathogen-associated molecular pattern
PB	Basic polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PI	Propidium iodide
PLY	Pneumolysin
PPV23	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
PS	Phosphatidylserine
QIV	Quadrivalent influenza vaccine
qPCR	Quantitative polymerase chain reaction
RAR	Retinoic acid receptor
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid

RORC	RAR-related orphan receptor C (RORC)
RLR	RIG-I-like receptor
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RV	Rhinovirus
RVI	Respiratory viral infection
SEM	Standard error mean
S.p.	*Streptococcus pneumoniae*
TCID	Tissue culture infectious dose
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TICAM1	TIR-domain-containing adaptor protein inducing IFN-β
TICAM2	TRIF-related adaptor molecule
TIR	Toll IL-1 Receptor
TIRAP	MyD88-adaptor-like
TIV	Trivalent influenza vaccine
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
UV	Ultraviolet
vRNP	Viral ribonucleoprotein particles

UNITS

%	Percentage
°C	Degrees Celsius
CFU	Colony forming units
g	Grams
hr	Hours
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
n	Number
ng	Nanogram
nm	Nanometre
U	Units
x g	Gravitational force
s	Second
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

TABLES

FIGURES

PUBLICATIONS

Maguire, P.T., Loughran, S.T., Harvey, R., Johnson, P.A. A TLR5 mono-agonist restores inhibited immune responses to *Streptococcus pneumoniae* during influenza virus infection in human monocytes. In submission to Journal of General Virology.

McQuaid, S., Loughran, S., Power, P., **Maguire, P.**, Szczygiel, A., Johnson, P. Low dose IL-2 induces $CD56^{bright}$NK regulation of T cells via NKp44 and NKp46. In submission to Clinical and Experimental Immunology (CEI-2019-8012).

Loughran, S.T., Power, P.A., **Maguire, P.T.**, McQuaid, S.L., Buchanan, P.J., Jonsdottir, I., Newman, R.W., Harvey, R., Johnson, P.A. 2018. Influenza infection directly alters innate IL-23 and IL-12p70 and subsequent IL-17A and IFN-γ responses to pneumococcus in vitro in human monocytes. *PLoS ONE* 13(9): e0203521. doi.org/10.1371/journal.pone.0203521.

McQuaid, S., Loughran, S., Power, P., **Maguire, P.**, Walls, D., Grazia Cusi, M., Orvell, C., Johnson, P. 2018. Haemagglutinin-neuraminidase from HPIV3 mediates human NK regulation of T cell proliferation via NKp44 and NKp46. *J. Gen Virol*. 99(6):763-767. doi: 10.1099/jgv.0.001070.

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Sinéad Loughran, Patrick Power, **Paula Maguire**, Samantha McQuaid, Richard Lalor, Ingileif Jonsdottir, Robert Newman, Patricia A. Johnson. Influenza and Secondary Bacterial Infections: How to Tackle Antimicrobial Resistance? School of Nursing and Human Sciences Research Expo 2016, Dublin City University. Dublin, 23rd November 2016.

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ABSTRACT

The role and mechanism of immunomodulation by influenza virus and its components in the predisposition to bacterial disease during influenza infection

Paula Maguire

Influenza A virus (IAV) infection predisposes individuals to severe infections with bacteria such as *Streptococcus pneumoniae* (*S.p*.). Research shows that influenza infection impairs the T helper 17 (Th17) immune response, which is critical in the clearance of *S.p.* infections. Studies have demonstrated a role for type I Interferons in the impaired Th17 immunity associated with IAV. The results presented in this thesis demonstrate that IAV infection significantly impairs *S.p*. driven innate and adaptive cytokines. However, this inhibition occurred in the absence of type I Interferons, suggesting an additional mechanism of Th17 immunomodulation associated with IAV. To establish how IAV inhibits these responses, we investigated the effect of IAV infection on specific innate immune Toll Like Receptors (TLRs), which are triggered by *S.p.* infection. We have identified that IAV targets TLRs (TLR2, TLR4, TLR9) in human monocytes, resulting in a reduction in TLR-induced cytokines. The effect of IAV is more profound on the TLR2 and TLR9 pathways. We established IAV may be inhibiting the TLR9 pathway by targeting RORC, a Th17-specific transcription factor. We investigated if TLR5 agonism could restore IAV-inhibited immune responses. Levels of pneumococcus driven cytokines, which had previously been inhibited by IAV were not reduced in the presence of the TLR5 agonist, suggesting this may restore immune responses despite IAV inhibition. Finally, we sought to investigate the role of the influenza surface glycoprotein, haemagglutinin (HA) in innate and adaptive responses to *S.p*. and innate responses to TLR agonism. Pneumococcus driven innate and adaptive cytokines were significantly inhibited by HA, whilst certain TLR agonist driven cytokines were also inhibited by HA. Novel findings include determining that immune inhibition by IAV is not solely due to type I IFNs, and demonstrating that TLR5 agonism may be beneficial in circumventing immune inhibition by IAV and restoring Th17 responses.

1.0 GENERAL INTRODUCTION

1.1 OVERVIEW

The overall aim of this project is to examine the effect of influenza A virus (IAV) infection on human immune responses to *Streptococcus pneumoniae* (*S.p.*). It has long been established that IAV infection predisposes individuals to secondary infections, particularly with capsular, extracellular bacteria such as *S.p*. [1–3]. These secondary infections can often prove fatal to both immunocompromised individuals and previously healthy individuals [4,5]. The mechanisms as to why this synergy occurs are not clearly understood.

Vaccine administration is the primary mode of prevention of influenza infections, however these must be reformulated annually due to the virus constantly mutating [6,7]. Furthermore, the effectiveness of vaccines varies year by year (approximately 30-60% in Europe), therefore despite vaccine improvement, comprehensive prevention of influenza infection is not currently possible [8–13]. As influenza infections will continue to commonly occur, so too will secondary bacterial infections. Due to the high prevalence of secondary bacterial infections associated with IAV infections, patients are often prophylactically prescribed antibiotics upon presenting with viral infections such as influenza [14]. However, this is contributing to the considerable problem of antimicrobial resistance that we are already facing. Drug-resistant infections are increasing rapidly, therefore new treatment options must be explored [15].

The majority of research in this area has been carried out in animal models (including mice, ferrets, and guinea pigs), resulting in a deficit of research in human models. In an attempt to address this gap in the research, a human *ex vivo* model was used throughout the project. Key cytokines involved in the response to *S.p.* were chosen to analyse and differences in response to IAV infections were investigated. To provide mechanistic insights into how IAV inhibits responses to *S.p.*, possible indirect and direct modes of inhibition were examined, including the role of type I IFNs, and key Toll Like Receptors (TLRs) involved in *S.p* responses. The effect of IAV on TLR-induction of the Th17-specific cytokine, retinoic acid receptor (RAR)-related orphan receptor C (RORC) was determined to examine possible molecular mechanisms behind IAV inhibition. An additional IAV component (haemagglutinin), was examined to further explore possible modes of inhibition by IAV.

1.2 INFLUENZA VIRUS

The influenza virus is a respiratory virus belonging to the *Orthomyxoviridae* family of negative sense RNA viruses [16]. Influenza is a highly contagious, airborne disease and can be transmitted via direct/indirect contact, droplets, and aerosols [17], meaning the virus can spread easily from individual to individual. Influenza virus can cause both epidemics and pandemics [18]. Typical symptoms of influenza infection include high fever, cough, nasal inflammation, fatigue, and muscle pain [19,20]. However, serious complications can occur both in healthy individuals and within at-risk age groups, immuno-compromised individuals, and those with underlying conditions such as asthma [21]. Additionally, there is a huge economic burden due to influenza infection. In the United States alone, it is estimated that the average annual total economic burden of influenza was $11.2 billion [22]. Due to both health and economic reasons, controlling and preventing viral infections such as influenza has been a major area of research. The research has focused primarily on development of both vaccines and antiviral agents. In the 2009 influenza pandemic, neuraminidase inhibitors (NIs) were the major type of influenza antivirals used [23,24]. As these are merely a treatment and not a means of preventing influenza infection, vaccines are considered to be the most beneficial tactic. Most vaccines are inactivated and injectable [25]. Individuals must be vaccinated annually due to the ability of the influenza virus to mutate rapidly and older individuals require a stronger dose of haemagglutinin in trivalent vaccines in order to achieve immunity [26]. However, current vaccines will not be sufficiently beneficial in the event of an influenza pandemic and in the time it would take to develop a vaccine for a pandemic strain, millions of people could die. Therefore, it is of paramount importance that the immune responses to influenza infection are more clearly understood in order to develop better treatment strategies.

There are four distinct classifications of influenza virus: Influenza A, Influenza B, Influenza C, and Influenza D [27]. The classifications are based on the antigenic specificity of their envelope proteins. Influenza A virus is the major type in circulation in humans as well as in birds, dogs, pigs, and horses, and is the focus of this study [28].

1.2.1 INFLUENZA A VIRUS

Influenza A Virus is a respiratory virus, which is highly contagious and poses substantial public health risks due to its strong association with morbidity and mortality [18]. Global pandemics are notorious, for example in the 1918 pandemic, it is estimated that

approximately 50-100 million respiratory deaths occurred [29]. Current estimates state that 290,000-650,000 deaths are caused by seasonal influenza virus annually [30,31].

1.2.1.1 Structure of Influenza A Virus

Influenza A virus is comprised of a spherical, protein shell called a capsid, surrounded by a lipid envelope which forms the virion. Within the virion, there are eight separate segments of single-stranded RNA which each encode either one or two discrete proteins (Figure 1.1) [28,32]. The gene segments are surrounded by the nucleoprotein (NP), at the end of which are the polymerase proteins, basic polymerase 1 and 2 (PB1 and PB2) and acidic polymerase (PA). Basic polymerase 1 is involved in addition of nucleotides during nucleic acid synthesis. Basic polymerase 2 controls host-cell RNA recognition, and PA is thought to be involved in transcriptase protease activity [33]. Together, these form the viral ribonucleoprotein particles (vRNPs), which move to the host cell nucleus and are responsible for the initiation of viral transcription and replication of viral RNAs [16]. Two of the genomic segments encode two different surface glycoproteins called haemagglutinin (HA) and neuraminidase (NA) (Figure 1.1), which act as viral antigens [33]. There are 18 different HA subtypes and 11 different NA subtypes, which determine the subtype of the influenza virus based on the type of HA and NA expressed [34–36]. The main role of HA is to bind to sialic-acid receptors and to mediate entry into host cells. NA cleaves sialic acids and is also involved in budding of new virions from infected cells. Different segments also encode for NP, the matrix proteins (M1 and M2), non-structural protein 1 (NS1) and non-structural protein 2 (NS2) (also known as nuclear export protein (NEP)). Nucleoprotein binds to viral RNA, whereas M1 and M2 encode the primary element of the virion and function as an ion channel, respectively (Figure 1.1). NS1 is encoded by the smallest segment in the viral genome and it is involved in RNA transport, splicing, and translation [33]. Additionally, NS1 inhibits nuclear export of host cellular mRNA by impeding cleavage of host mRNA at the polyadenylation cleavage site [37]. Viral mRNAs are not negatively affected by NS1 as their poly(A) tails are not generated by the host machinery, but are synthesised by the viral RNA polymerase [38,39]. NS2/NEP is encoded by the same segment as NS1, and was initially thought to have no structural function within the virion [40]. However it was subsequently found to interact with M1 [41], and to play a role in the export of vRNPs from the nucleus of the host cell [42].

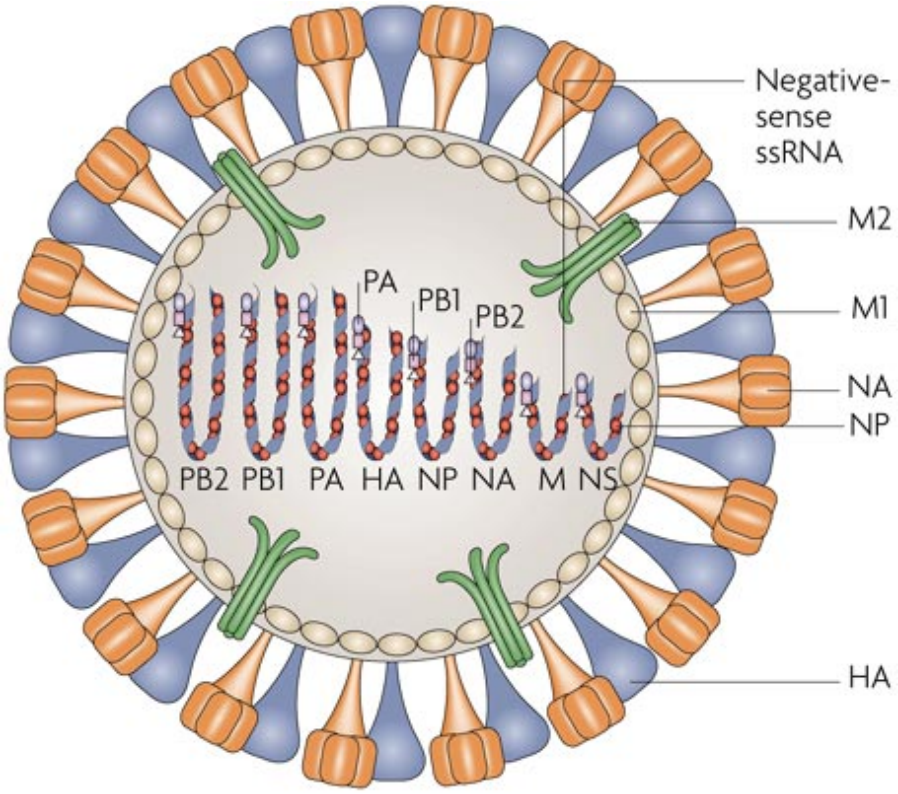

Figure 1.1 The structure of Influenza A Virus. Image taken from Nelson and Holmes, 2007 [33].

The nomenclature for influenza virus uses the following system as approved by the World Health Organisation in 1980 (Figure 1.2) [43]:

- Antigenic type (A, B, C, D)
- Host of origin (For non-human origin only)
- Geographical origin (Taiwan, Panama etc.)
- Strain number
- Year of isolation
- For influenza A viruses, haemagglutinin and neuraminidase antigen description in parentheses (H1N1)

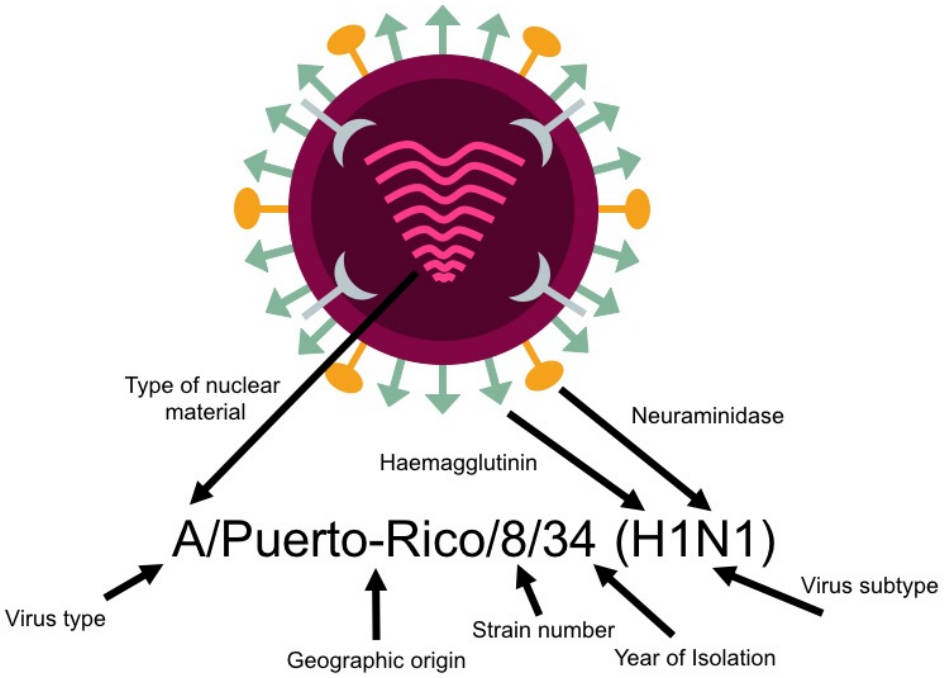

Figure 1.2 Classification and nomenclature of Influenza A Virus

1.2.1.2 Life Cycle of Influenza Virus

The life cycle of the influenza virus can be divided into different stages: entry into the host cell, entry of vRNPs into the nucleus, transcription and replication of the viral genome, export of the vRNPs out of the nucleus, and viral budding [44]. The influenza virus HA binds to receptors containing sialic acid on the surface of the host cell, which initiates viral infection and subsequently triggers endocytosis. The HA component is composed of two subunits: HA1 and HA2, which are linked by disulphide bonds [45]. HA1 contains the receptor binding domain, and HA2 contains the fusion peptide [45]. The sialic acids on the cell membranes are bound to carbohydrates via two major linkages: α(2,3) and α(2,6). These linkages play a significant role in the HA binding specificity. Viruses adapted to infect humans mostly recognise the α(2,6) linkages, whereas viruses affecting birds and horses mostly recognise the α(2,3) linkages, and viruses affecting pigs recognise both [46]. The low pH within the endosome of the host cell, where molecules may be sorted for degradation, causes conformational changes of the HA molecule resulting in fusion of the viral and endosomal membranes [46]. Additionally, the acidity of the endosome causes a conformational change of the M2 component causing it to function as an ion channel, which leads to acidification of the virion interior. The acidity releases vRNPs from the M1 component, which can enter the cytoplasm of the host cell (Figure 1.3) [47]. The vRNPs are subsequently transported to

the nucleus, where the viral RNA is transcribed and replicated by the viral RNA polymerase. As influenza RNA is composed of negative sense strands, it must initially be converted into positive sense RNA to be transcribed [48]. The PB2 viral component binds to the 5' methylated cap of the host cell messenger RNA (mRNA) and the PA component of the virus, which has endonuclease activity, cleaves the host cell mRNA 10-13 nucleotides downstream from the 5' methylated cap [49–51]. Transcription of the viral mRNA is initiated from the cleaved 3' end of the RNA fragment [51]. This process is known as "cap-snatching". Six of the viral segments encode for a single transcript, and two (segments 7 and 8) encode for two transcripts each via alternative splicing. Segment 7 encodes for M1 and M2, and segment 8 encodes for NS1 and NS2/NEP [40,52]. The new viral mRNA is exported back to the cytoplasm for translation into viral proteins. M1 and NS1/NEP play an important role during nuclear export [53]. The viral surface proteins HA, NA, and M2 are translated in the endoplasmic reticulum (ER), glycosylated in the Golgi apparatus, and transported to the host cell membrane. Concurrently, the NS1 protein prevents the host from producing host mRNAs, resulting in the inhibition of anti-viral interferon-β (IFN-β) [38,54]. Once released from the nucleus, newly formed vRNPs form progeny virions at the cell membrane. The new virions are released from the cell by budding via cleavage of sialic acid residues by NA, and can go on to infect neighbouring cells [55].

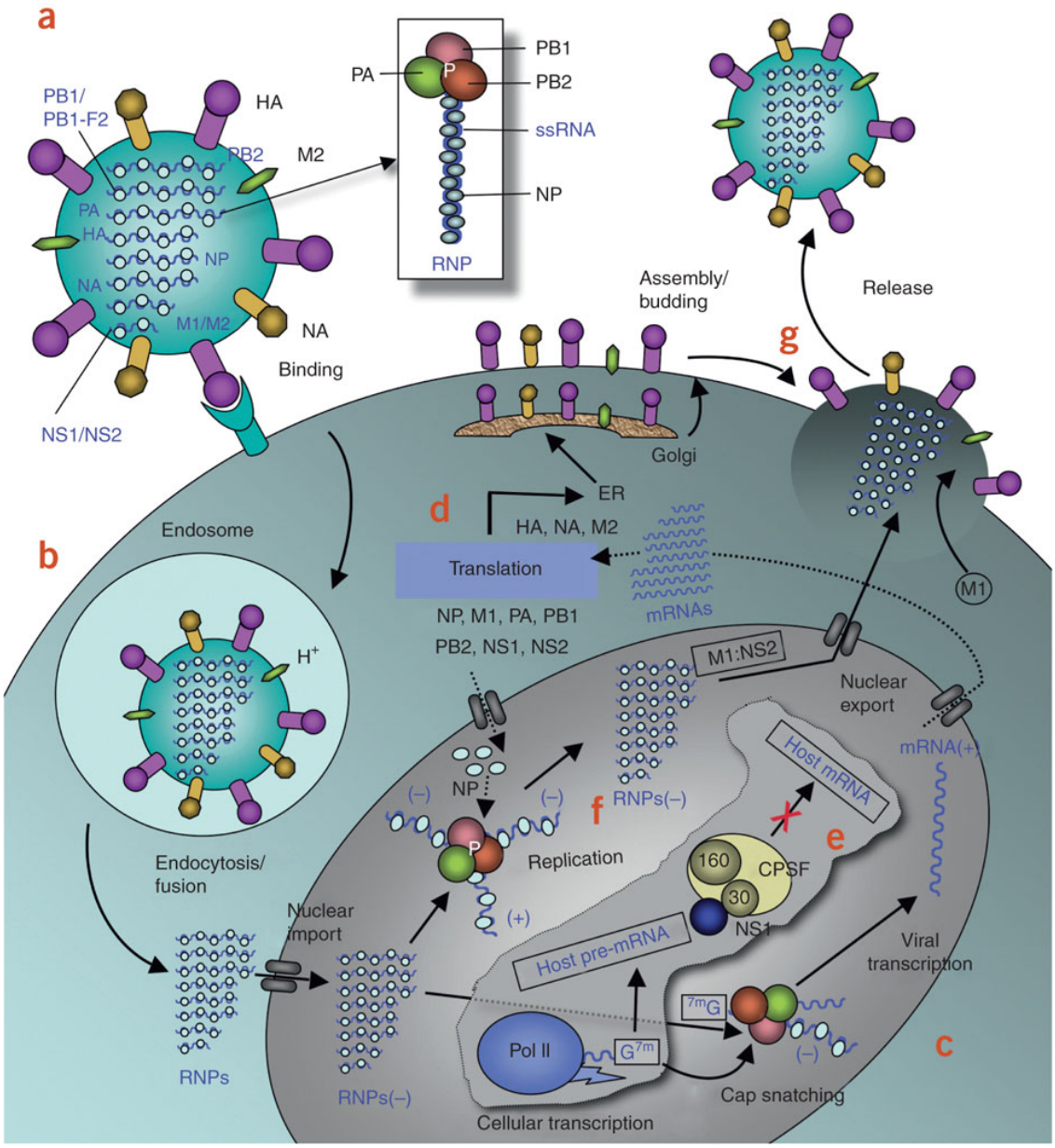

Figure 1.3 Life cycle of the Influenza virus. (a) Influenza A Virus haemagglutinin binds to host cell-surface sialic acid receptors and the virus is transported into the host cell, (b) where it is endocytosed causing vRNPs to be released into the cytoplasm. (c) The vRNPs are transported into the nucleus, where viral mRNA synthesis and transcription occur. (d) Viral mRNAs are transported back to the cytoplasm and translated into viral proteins. (e) The protein, NS1 inhibits the host from producing mRNAs such as Interferon-β. (f) The vRNPs are transported back to the cytoplasm and (g) once they reach the cell membrane, are integrated into new virus which are budded out and released from the host cell. Image taken from Das et. al., 2010 [56].

1.2.1.3 Pathogenesis of Influenza Virus

Influenza virus infects epithelial cells, causing functional changes in the respiratory tract, such as decreased oxygen exchange [57]. Many studies have demonstrated a link

between severe influenza infection and a strong activation of host inflammatory responses, such as high cytokine and chemokine expression in both humans and animals [58,59]. Typical clinical symptoms of influenza infection include sudden onset of fever, nasal inflammation, cough, headache, fatigue, muscle pain, and inflammation of the trachea and upper respiratory tract [19,20,60]. While acute symptoms and fever usually subside after 7-10 days, fatigue can persist for weeks [60]. Complications arising from influenza infection include bronchitis and pneumonia, which may result in death, especially in immunocompromised individuals [60–62]. Additionally, influenza infection predisposes patients to secondary bacterial infections [63–65].

1.2.1.4 Treatment and Prevention of Influenza Virus Infections

Neuraminidase inhibitors (NIs) such as osteltamivir and zanamivir are a commonly used treatment of influenza infection [66,67]. Osteltamivir is an oral solution or capsule and is the primary drug used. It is usually prescribed only to those in an “at-risk” group such as pregnant and immunocompromised individuals [68]. The anti-viral properties of osteltamivir are most effective when administered within 48 hours of onset of symptoms [69]. Resistance to osteltamivir has been observed in H1N1 infections, but not in H3N2 infections [70,71]. Where resistance does occur, zanamivir is prescribed. Zanamivir is administered via inhalation, and has not yet been approved in children younger than five years old [72]. Zanamivir treatment should be initiated within 36 hours of onset of symptoms [69]. Early administration of anti-viral drugs such as NIs can reduce the risk of complications from influenza infection [69,73,74], although NI treatment after 48 hours of onset of symptoms can still be beneficial if the patient has severe complications [75,76]. Although NIs can be very effective at reducing both the severity and symptom duration of influenza infections, serious adverse side-effects have been reported in recent years [77]. Such reactions include nausea, hypothermia, and neuropsychiatric reactions such as hallucinations, which appear from less than 1 hour after initiation of treatment [77–79], or delayed reactions such as renal, immune and psychiatric disorders, which tend to appear at least a few days after initiation of treatment [78].

Vaccination is the principal tactic in the prevention and regulation of influenza infections, but due the constant mutating virus, vaccines must be reformulated annually [6,7]. The seasonal influenza vaccine is formulated to protect against the three or four strains of influenza virus most likely to be in circulation during a particular year [25]. Influenza vaccines can be administered as an intramuscular or subcutaneous injection which contains an inactivated form of the virus, or as a nasal spray containing a live but

attenuated version of the virus [25]. There are three types of influenza vaccine available: an inactivated trivalent influenza vaccine (TIV) which contains antigens from two IAV and one influenza B virus (IBV) strain; an inactivated quadrivalent influenza vaccine (QIV) which contains antigens from two IAV and two IBV strains; and a live attenuated quadrivalent influenza vaccine (LAIV) [80]. The level of efficacy of influenza vaccines varies annually; in Europe, there is approximately a 30-60% rate of vaccine efficacy [8–13]. Additionally, older individuals require a stronger dose of haemagglutinin in trivalent vaccines in order to achieve immunity [26].

1.2.1.5 Antigenic Shift and Drift

Influenza is a recurring disease that re-emerges annually in a different configuration. The influenza virus has high mutation rates ($1x10^{-3}$ - $8x10^{-3}$ substitutions per site annually) because the RNA polymerase lacks proof-reading ability [81]. These mutations result in amino acid alterations in the surface proteins HA and NA via processes known as antigenic drift and antigenic shift [82]. Antigenic drift occurs due to a small degree of RNA mutation and results in a minor change in a single virus [83]. This process causes annual influenza epidemics, resulting in millions of human infections due to the emergence of new virus strains (Figure 1.4). Antigenic shift, however is a process which causes major antigenic changes. It occurs during simultaneous infection of a cell with two different influenza viruses, resulting in a mixing or re-assortment of the genes between the different subtypes leading to a new virus which possesses a mixture of proteins from the original viruses (Figure 1.5). This process leads to influenza pandemics which occur due to the emergence of new viral subtypes. Infections may be spread between different species and are difficult to treat. The major changes to the surface proteins mean that the new influenza virus variations can avoid immune detection and invade host cells [84]. The mutations are also the reason why vaccines must be reformulated every year [6,7].

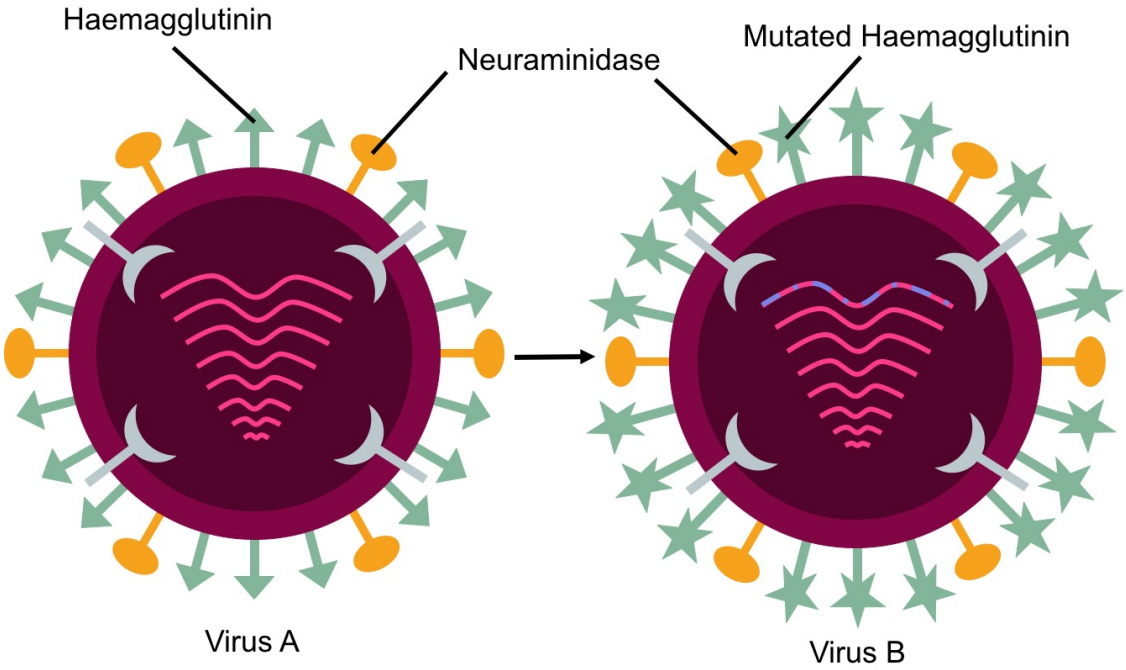

Figure 1.4 Antigenic drift in influenza virus. In antigenic drift, genetic mutations in haemagglutinin and/or neuraminidase result in small antigenic changes over time.

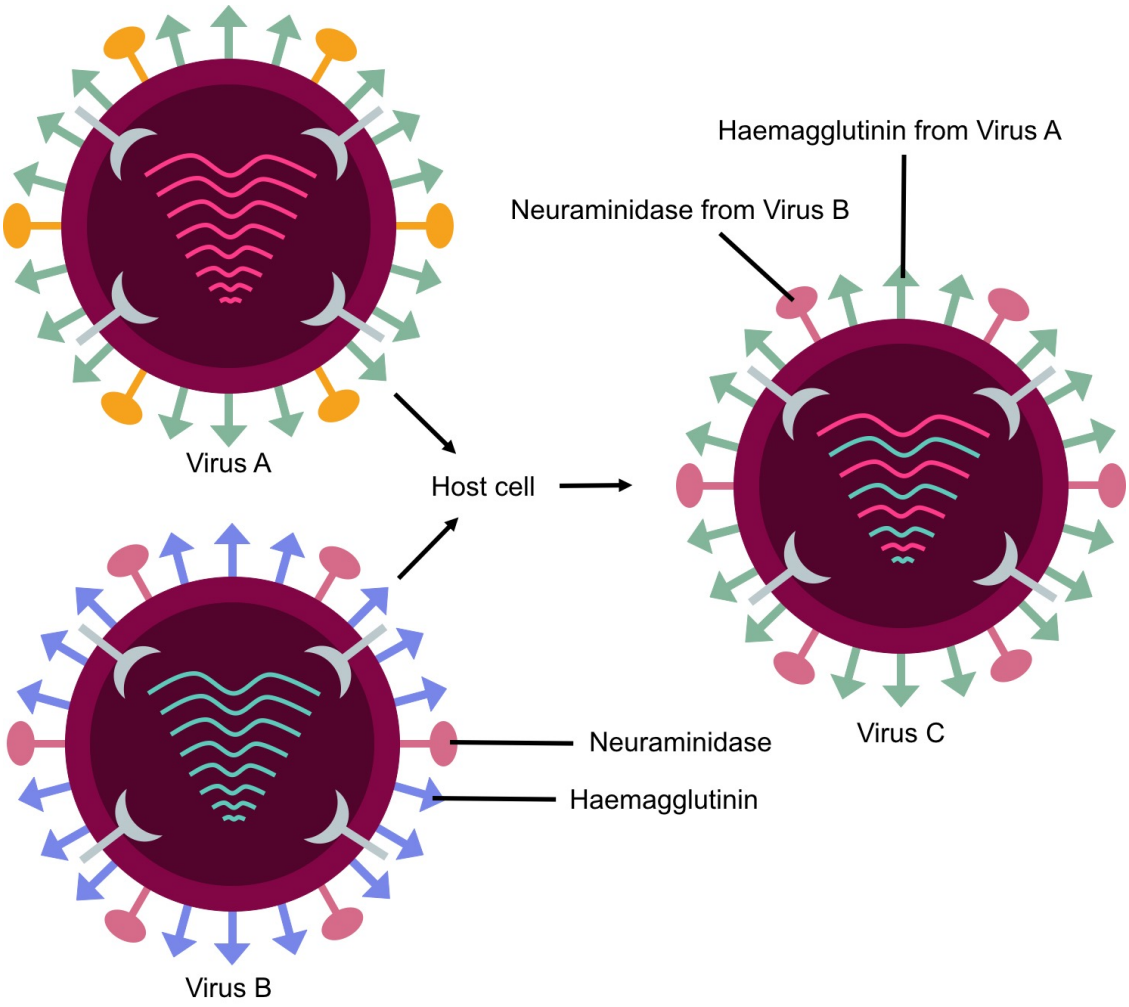

Figure 1.5 Antigenic shift in influenza virus. In antigenic shift, during simultaneous infection with two different influenza viruses, the genes from the different viruses can mix, resulting in a new variant which contains a combination of proteins from the original viruses.

1.3 SECONDARY BACTERIAL INFECTIONS

As mentioned previously, approximately 290,000-650,000 deaths are caused by seasonal influenza virus annually [30]. Most of these deaths are due to secondary bacterial pneumonia, which may result in superinfection [18,85]. There is considerable evidence from animal models, and clinical data that IAV infection predisposes individuals to bacterial infection typically with capsular, extracellular bacteria such as *S.p*. and *Staphylococcus aureus* [1–3]. In the 1918 pandemic, the late 1960's pandemic, and the 2009 pandemic, the predominant bacterial co-pathogen was *S.p.* [20,86,87]. Globally, in seasonal IAV, the most common co-infecting bacteria is also *S.p*. [18]. It is widely believed that these co-infections are a major contributor to increased morbidity and mortality associated with both seasonal and pandemic outbreaks of influenza [18]. Influenza-bacterial co-infection can result in hospitalisation and/or death of both patients with pre-existing lung disease and previously healthy individuals [4,5]. Although a considerable effort has been made over the last decade, no real consensus has been reached as to why these secondary infections occur. Earlier studies have suggested that physiological damage to the respiratory epithelium, and increased adherence factors for *S.p* may be involved [1]. While these processes are likely to contribute to more enhanced colonisation, recent research has pointed to the role of immunological mechanisms in the susceptibility to invasive bacterial disease during influenza infection [1,88,89]. Impaired immunological responses have been suggested as contributing to susceptibility, such as reduced responsiveness of alveolar macrophages and elevated levels of anti-inflammatory IL-10 in mice [89–91]. Also, a neutrophil influx caused by viral and bacterial toxins may result in a cytokine storm leading to a destructive hyper inflammatory response in mice [92].

1.3.1 STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae (also commonly referred to as pneumococcus) is a major human pathogen of global significance. Worldwide, between 2006-2016, *S.p* was the primary cause of lower respiratory infection morbidity and mortality, with approximately 1,189,937 deaths being attributed to the pathogen in 2018 alone [93,94]. *Streptococcus pneumoniae* was also the leading cause of community-acquired pneumonia, which can lead to invasive pneumococcal disease such as septicaemia, bacteraemia, pneumonia and meningitis [95,96]. There have been more than 90 distinct serotypes of *S.p* discovered thus far. Certain serotypes are linked with higher mortality, such as serotype

3 [97]. The different serotypes are characterised by a discrete polysaccharide capsule. The polysaccharide capsule acts as an important virulence factor mainly via inhibition of phagocytosis [98]. The cytotoxic endotoxin, pneumolysin (PLY) is also a key virulence factor as it forms large pores in the membrane of mammalian cells [99]. Pneumolysin has been shown to be a vital component in the ability of *S.p.* to cause disease such as pneumococcal pneumonia, and meningitis, but may not be necessary for the advancement of secondary pneumococcal pneumonia following influenza virus infection [100–102]. Upon entering the nasal cavity, *S.p.* utilises the polysaccharide capsule to reduce entrapment in the mucus secretions, which allows *S.p.* access to the surface of the epithelium [103]. *Streptococcus pneumoniae* also produces enzymes, which include NA. The NA enzyme cleaves terminal sugars on host glycoproteins and glycolipids, which exposes receptors and aids bacterial adherence [104,105]. Colonisation of *S.p.* occurs most frequently in early childhood, with most infants carrying at least one serotype, either simultaneously or consecutively [104]. By adulthood, the rate of *S.p.* colonisation declines to below 10%, indicating that young children are the main carriers of *S.p*. [106].

1.3.2 TREATMENT OF STREPTOCOCCUS PNEUMONIAE INFECTIONS

Streptococcus pneumoniae infections are often treated with antibiotics such as amoxicillin, doxycycline and penicillin. Resistance to penicillin had become very common, however with the introduction of *S.p.* vaccines, resistance to penicillin began to decrease, before increasing again [107]. There are two distinct pneumococcal vaccines: the pneumococcal conjugate vaccine (PCV) and the pneumococcal polysaccharide vaccine (PPV23). The PCV is administered to all babies as part of the childhood immune schedule, and the PPV23 is administered to individuals over the age of 65 and those with certain medical conditions. The PPV23 protects against 23 different types of pneumococcal disease [108]. Vaccinating young children has been shown to result in “herd immunity”, which has benefitted those of all ages who do not receive the vaccine themselves [106]. Despite this, treatment of *S.p.* infections are becoming continually more complex due to *S.p* serotypes diverging from those covered by the pneumococcal vaccine and the emergence of antibiotic resistant strains of *S.p.* The increase in antimicrobial resistance (AMR) is an important clinical manifestation of particular concern [15]. Due to the high incidence of secondary bacterial infections associated with IAV, often patients are prophylactically prescribed antibiotics upon

presenting with viral infections [14]. Prophylactic administration of antibiotics is adding to the overall problem of AMR and should be addressed.

1.3.3 ANTIMICROBIAL RESISTANCE

Antimicrobial medicines are active against infections caused by a multitude of pathogens. Antimicrobial resistance occurs when the pathogens which cause infections survive exposure to a treatment that usually would kill them or prevent their growth. This enables the pathogens which have survived to spread. Antimicrobial resistance is one of the biggest health threats that humans currently face. Currently, approximately 700,000 deaths every year are due to AMR, however it has been estimated that by 2050, the number of predicted deaths attributed to AMR will increase to 10 million annually, surpassing current deaths attributed to cancer and diabetes combined (Figure 1.6) [15]. Additionally, there are huge economic effects, as 100 trillion US dollars of economic output will be exposed due to the increase of antimicrobial-resistant infections, unless pre-emptive steps are taken to impede the increase in drug resistance. In the U.S., it costs the health system 20 billion US dollars as over two million bacterial infections are caused by strains which are resistant to the first-line antibiotic drug treatment [109]. Antibiotics are pivotal to a functioning healthcare system. If antibiotics continue to lose efficacy, fundamental medical procedures such as caesarean sections, joint replacements, organ transplants, and even chemotherapy may become too risky to perform [110]. Drug-resistant infections have typically been associated with hospitals, but more recently these infections have been observed in the wider community [111]. To prevent the global increase in drug-resistant infections, new treatments must become available to cope with the decrease in the efficacy of older medicines. Additionally, the mis-use of antibiotics should be addressed as over-prescribing of drugs and use of antibiotics in agriculture have been damaging. Many studies have detailed the increase in infections by antibiotic-resistant strains of *S.p.*, resulting in major clinical implications [112].

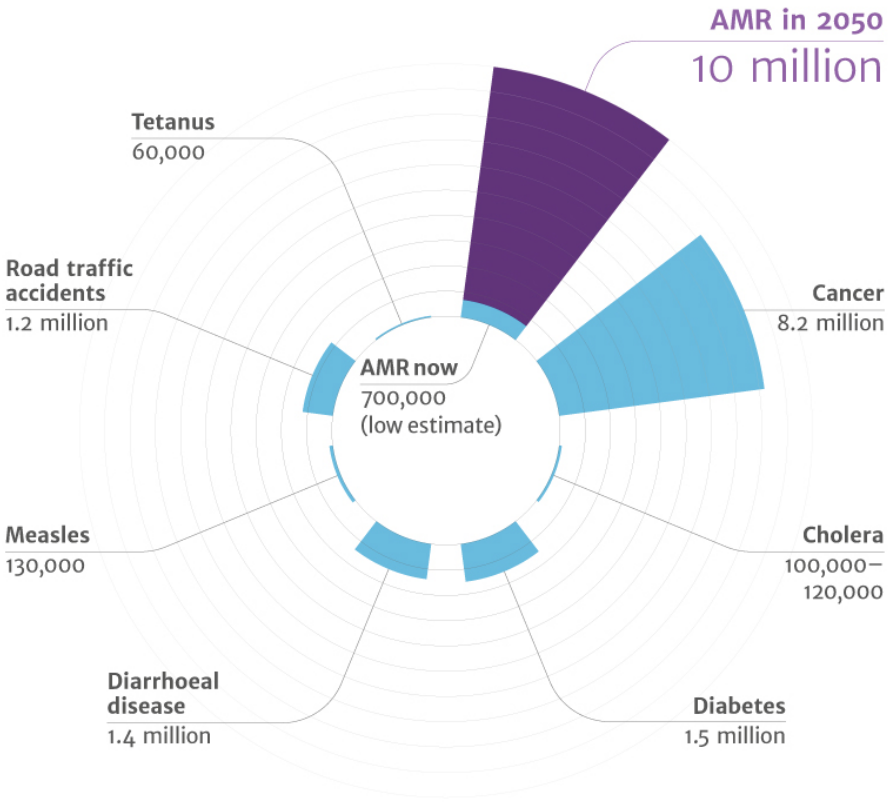

Figure 1.6 Predicted annual deaths attributable to Antimicrobial Resistance. Image taken from O'Neill, 2016 [15].

1.4 OVERVIEW OF THE HOST IMMUNE RESPONSE TO PATHOGENS

Humans are exposed to pathogens every day, through inhalation, ingestion, and contact. Our ability to avoid constant infections depends on the immune system. The immune system is highly complex and is critical to the outcome of infections. However, concurrently, pathogens are attempting to evade detection by the immune system. The immune response can be separated into two arms; the innate immune response, which is rapid, but non-specific, and the adaptive immune response, which is slower, but very specific. The innate and adaptive immune responses work together and complement each other to provide the fastest and most effective immunity [113].

1.4.1 THE INNATE IMMUNE RESPONSE

The innate immune response is the body's first line of defence against pathogens including influenza and *S.p.* [16]. This response is critical, especially during the first few hours after exposure to a new pathogen. Innate immune responses are not specific to any particular pathogen, but can be mounted very rapidly. The innate immune response functions due to proteins and phagocytic cells which recognise conserved pathogenic features. The initial lines of innate defence are the physical and chemical barriers, such as the epithelial surface, mucosal membranes, and digestive enzymes. If the physical and chemical barriers fail to prevent the pathogen from invading further, then the pathogen will next encounter the innate immune cells, including numerous leucocytes, such as monocytes and natural killer (NK) cells [113]. These cells express receptors, often called pattern-recognition receptors (PRRs), which are germline-encoded and recognise highly conserved microbial molecules or pathogen-associated molecular patterns (PAMPs) which are essential for pathogen survival [114]. Examples of PAMPs include peptidoglycan, flagella, lipopolysaccharides (LPS), unmethylated CpG motifs, and teichoic acid, all of which are found in bacteria, along with dsRNA produced in virus-infected cells [113,114]. In an influenza infection, the first innate barrier is the mucous layer, and once this layer is broken through, the virus targets respiratory epithelial cells. Upon entering the host cell, the virus is recognised by innate receptors which trigger signalling cascades resulting in the production of effector molecules which confer protection against the pathogen. Cells such as neutrophils, macrophages, and dendritic cells are early responders and are among the first line of cellular defence against the infection [115].

1.4.1.2 Cells of the Innate Immune System

Innate immune cells are not specific to any pathogen, and are mounted very rapidly. Immune cells originate from hematopoietic stem cells in the bone marrow. Hematopoietic stem cells produce two specialised types of stem cell: lymphoid progenitor cells and myeloid progenitor cells. Lymphoid progenitor cells produce T cells, NK cells, and B cells, whereas myeloid progenitor cells produce various types of leucocytes (white blood cells), as well as red blood cells and platelets. The different types of leucocytes are granulocytes (basophils, eosinophils, and neutrophils), mast cells, and monocytes. The granulocytes circulate in the blood stream and move into the tissues when recruited. Mast cells mostly function in allergic responses. Monocytes are one of the key innate immune cells. Once monocytes leave the bloodstream and enter the tissues, a subset of innate immune cells differentiate from monocytes to become either macrophages or dendritic cells (DCs) (Figure 1.7). These have effector functions similar to other cells of the innate immune system but perform the additional function of antigen presentation to the adaptive arm of immunity. Much attention has been paid to this group of immune cells as they link innate and adaptive immunity and are pivotal in defining the ultimate outcome of the infection [116].

Innate cells detect pathogens by cell-surface receptors, such as Toll Like Receptors (TLRs), which differentiate between the pathogen and the host. Once receptors signal the detection of a pathogen, cytokines are released. The generation of monocytes, macrophages and DCs depends on the receptor activation and subsequent cytokine release. The induced cytokines can either act in autocrine mode, by affecting the behaviour of the cell that has released the cytokine, or in paracrine mode, which affects the behaviour of adjacent cells. Monocytes, macrophages, and DCs secrete cytokines such as interleukin-1 (IL-1), IL-6, and IL-12 [113,116]. It is pivotal that the immune system can detect and destroy pathogens without harming the host. Therefore, the immune system must be able to distinguish between “self” and “non-self”. The innate immune response relies on PRRs recognising PAMPs which are not found on the host cell. The most common PRRs in mammalian cells are TLRs [114,117].

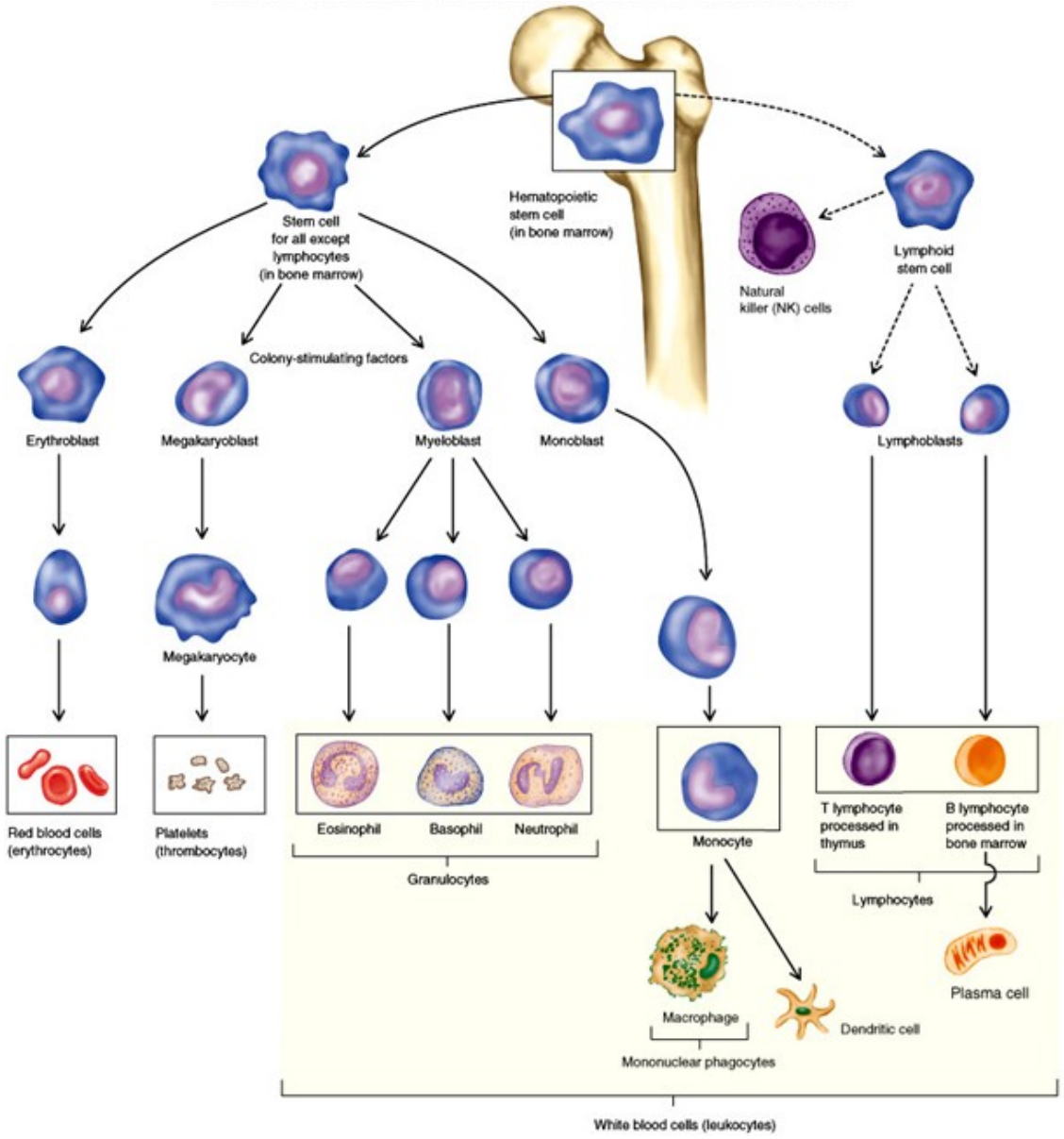

Figure 1.7 Cells of the immune system. Image taken from The McGraw-Hill Companies Inc., 2019 [118].

1.4.1.3 Pattern Recognition Receptors

Innate immune cells sense pathogens through innate receptors known as PRRs. These receptors include TLRs, which are located in the cellular membrane and endosome, and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which are located in the cytoplasm [16]. These receptors have two main functions: initiation of pathogen phagocytosis, and stimulation of host cell gene expression, which increases innate immune responses [113].

1.4.1.3.1 Toll Like Receptors

The Toll-like receptors are an essential group of PRRs [117]. They are named “Toll-like” as they are homologues of the *Drosophila* Toll protein [119,120]. There are ten different types of TLR in humans (TLR1-TLR10), with each responding to an array of PAMPs from a variety of microbes. Toll-like receptor 1, 2, 4, 5, and 6 are expressed on the cell surface, whereas TLR 3, 7, 8 and 9 are expressed intracellularly [121,122]. Toll-like receptor 1, 2, 4, 5, and 6 detect microbial components such as lipids, lipoproteins, and flagella. Toll-like receptor 3, 7, 8, and 9 detect both microbial and viral components [122]. Toll-like receptor 2 (along with TLR1 or TLR6), detects pneumococcal cell wall components such as lipoteichoic acid (LTA) and lipoproteins [123,124]. Toll-like receptor 3 detects viral dsRNA, while TLR4 detects pneumolysin (PLY) [125–128]. Toll-like receptor 5 detects bacterial flagellin, and TLR9 detects bacterial and viral DNA comprising unmethylated CpG motifs [129,130]. Toll-like receptors are type I integral membrane glycoproteins, which are characterised by extracellular domains. Each extracellular domain contains various amounts of leucine-rich-repeat (LRR) motifs, and a C-terminal cytoplasmic signalling domain, termed the Toll IL-1 Receptor (TIR) domains [131,132]. Stimulation of a TLR causes the TIR domain to recruit specific adaptor molecules such as innate immune signal transduction adaptor (MyD88), MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1), or TRIF-related adaptor molecule (TRAM, also known as TICAM2) (Figure 1.8). The recruitment of the adaptor proteins triggers downstream signalling cascades, which induces inflammatory cytokine and chemokine expression, anti-pathogen proteins, and initiation of the adaptive immune response [133]. It is the combination of adaptor molecules which decides the response to the pathogen [115]. All TLRs recruit MyD88, apart from TLR3, which uses TRIF. Toll-like receptor 2 uses MAL to recruit MyD88, whereas TLR4 can use MAL or TRAM to recruit either MyD88 or TRIF [134,135]. Once MyD88 is stimulated, it recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 [133,136]. Then, IRAK-1 associates with TNFR-associated factor 6 (TRAF6). MyD88-dependent and TRIF-dependent signalling pathways lead to the activation of nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3) or IRF7 through complexes which result in the production of anti-viral interferons and cytokine secretion [115,133]. In addition to sensing pathogens through TLRs, innate cells also play a crucial role in the activation of the adaptive immune response, such as macrophages and DCs which specialise in antigen presentation to T cells [113,116].

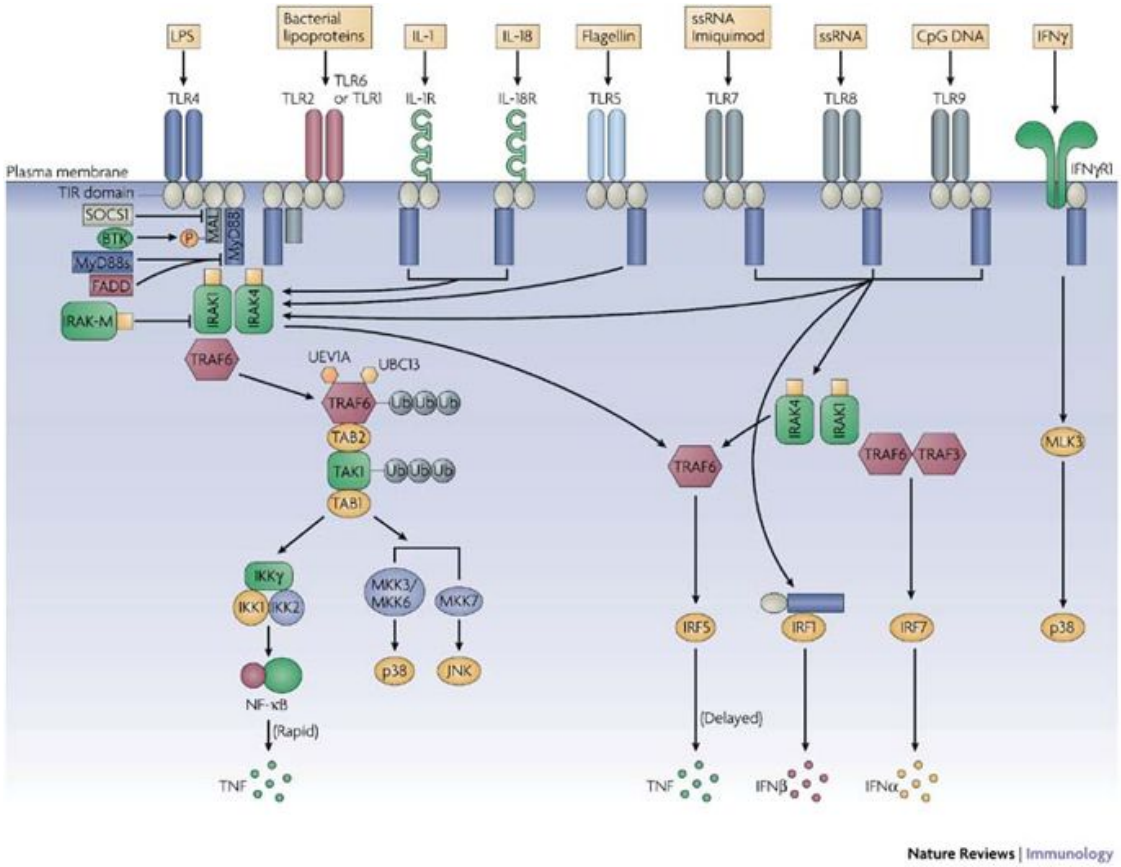

Figure 1.8 TLR signalling pathways. All TLRs, except TLR3, recruit MyD88 to the TIR domain, which in turn recruits IRAK4 and IRAK1. IRAK1 then associates with TRAF6 and TAK1, leading to NF-κB activation, cytokine secretion, and production of anti-viral interferons. Image taken from O'Neill, 2007 [133].

1.4.1.4 Antigen Presentation

Innate immune cells, such as DCs and macrophages, which differentiate from monocytes, sense pathogens through PRRs, however, they can also prime adaptive immune cells such as T cells to induce long-term immunity. T cells are distinct from other lymphocytes because of their T cell receptor (TCR), which contains two TCR chains (α and β), ζ-chain accessory molecule, and a CD3 co-receptor [116,137]. In a normal immune response, when a pathogen is sensed by the immune system, antigen-presenting cells (APCs) such as DCs and macrophages present antigens to naïve T cells via their major histocompatibility complex (MHC) class II molecules. MHC Class II molecules are usually only expressed on APCs and function by "trapping" peptide antigens from pathogens and displaying it for T cell recognition. The antigen being displayed by the APC is recognised by the TCR on the naïve T cell, however additional signals must be produced for the T cell to become activated [116]. A separate co-stimulatory signal must also be delivered by the APC to the T cell, usually by the B7

molecules (CD80/CD86) on the APC, which interact with the CD28 co-receptor on the T cell. This leads to T cell clonal expansion and depending on which innate cytokines are produced, differentiation into effector T cells such as T helper (Th) or T regulatory (T_{reg}) cells (Figure 1.9). Depending on the antigen presented, the co-stimulators and cytokines that have been produced, Th cells will differentiate into Th1, Th2, and Th17 effector cells. Each of which, produce their own range of cytokines and mediate distinct functions [116,137,138]. Th1 cells respond to intracellular pathogens and secrete the cytokine IFN-γ, amongst others. Th2 cells respond to parasitic infections, and Th17 cells respond to extracellular bacteria and secrete the pro-inflammatory cytokine, IL-17 [139–142]. T_{reg} cells are able to suppress innate and adaptive immune responses and can serve as a "self-check" to maintain immune homeostasis, primarily through the anti-inflammatory effects of IL-10 [137,143]. Ultimately, the innate immune response has a profound effect on the adaptive response which is subsequently elicited as APCs serve as the link between the innate and adaptive responses.

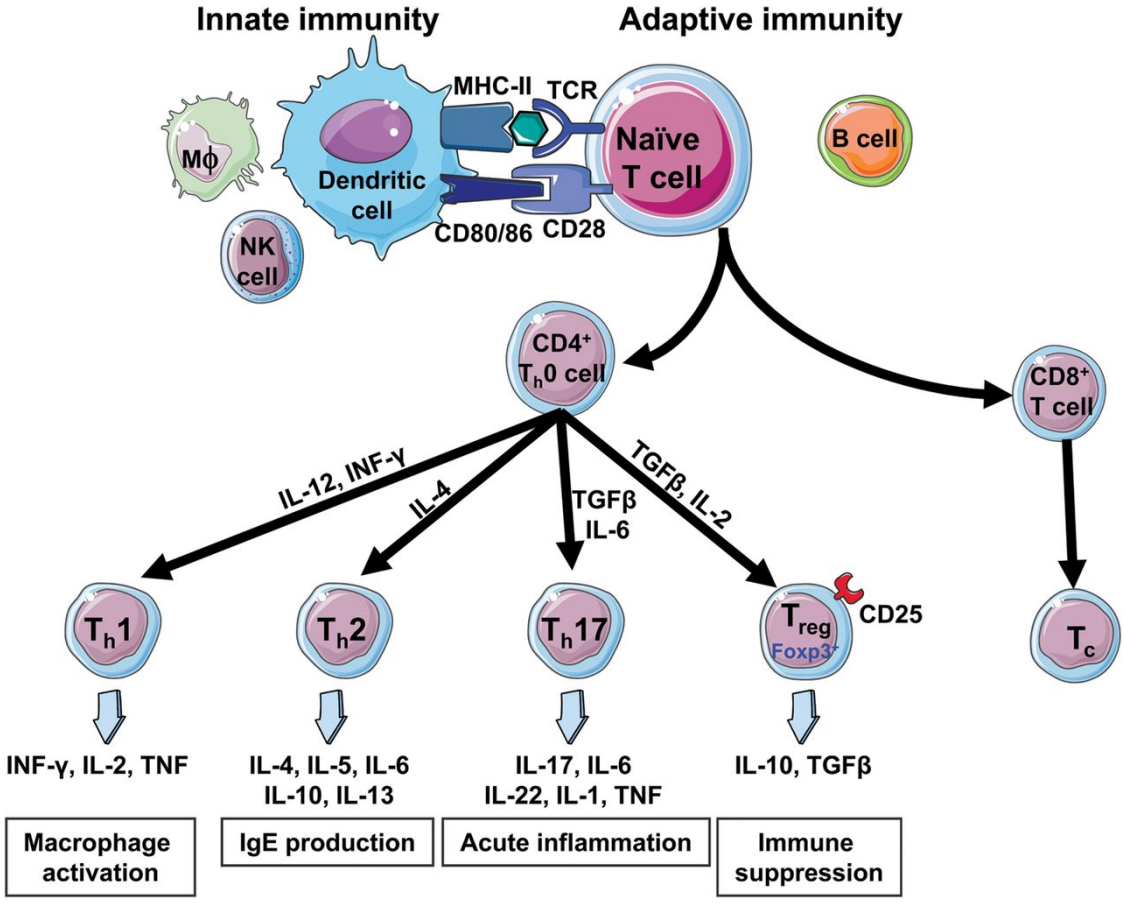

Figure 1.9 Differentiation of naïve T cell into Th cell subsets in a normal immune response. APCs present antigens to naïve T cells, leading to differentiation into effector T cells such as Th1, Th2, and Th17. Image taken from Idris-Khodja *et. al.,* 2014 [137].

1.4.2 THE ADAPTIVE IMMUNE RESPONSE

If the innate immune responses are unsuccessful in clearing infections, the adaptive immune response will be activated. The innate immune response is required to activate the adaptive immune response. Many of the same effector mechanisms are used in both the innate and the adaptive immune responses. The adaptive immune response is, however, much more specific in how the invading pathogens are targeted. The downside is that adaptive immune responses are slow to develop upon first exposure to a new pathogen, and can take at least a week before responses are effective, depending on the pathogen. Both the innate and the adaptive responses induce chemokines and cytokines to initiate inflammation. There are two arms of adaptive immunity: humoral immunity and cell mediated immunity. Humoral immunity is mediated by B cells, which mature in the bone marrow. Whereas cell mediated immunity involves T cells, which mature in the thymus. B cells express surface immunoglobulin receptors, and T cells express smaller antigen receptors. These distinct receptors allow B cells and T cells to execute different functions. Mature lymphocytes are constantly recirculating from the bloodstream through the spleen, lymph nodes, or mucosal lymphoid tissues, and back to the bloodstream via lymphatic vessels. The majority of adaptive immune responses are elicited when a recirculating T cell detects its specific antigen on the surface of an APC such as a dendritic cell or macrophage. As mentioned before, when a T cell detects an antigen, it proliferates and differentiates into antigen specific effector cells. In contrast, B cells proliferate and differentiate into antibody-secreting cells. The specificity of the immune response mounted by lymphocytes is made possible due to the receptors on the cell surface. The B cells express the B cell antigen receptor (BCR), whereas T cells express the T cell receptor (TCR) [113,116].

1.3.2.1 Humoral Immunity

After activation, the B cell secretes antibodies, which are identical to the BCR of the B cell. The B cell produces a considerable amount of antibodies in response to detection of an antigen. Antibodies are composed of two discrete regions: a constant region (Fc), and a variable region (Fab). There are five possible forms the constant region may take, whereas there is a very large number of forms the variable region may take. Antibody molecules are known as immunoglobulins. The two variable regions determine the antigen binding specificity of the antibody, whereas the constant region determines how the antibody destroys the pathogen once it has bound to it. Antibodies also contain two

identical heavy chains and two identical light chains (Figure 1.10). The heavy and light chains both contain variable and constant regions, with the variable regions of both the heavy and light chains combining to form the antigen binding site. Antibodies can protect the host in three ways: neutralisation, opsonisation, and complement activation. Production of antibodies are the only way B cells are involved in the adaptive immune response, whereas T cells have a diverse range of effector functions [113,116]. Influenza is primarily cleared from the body via humoral immunity through secretory IgA localised at the respiratory epithelium, and IgG in the serum [25,144].

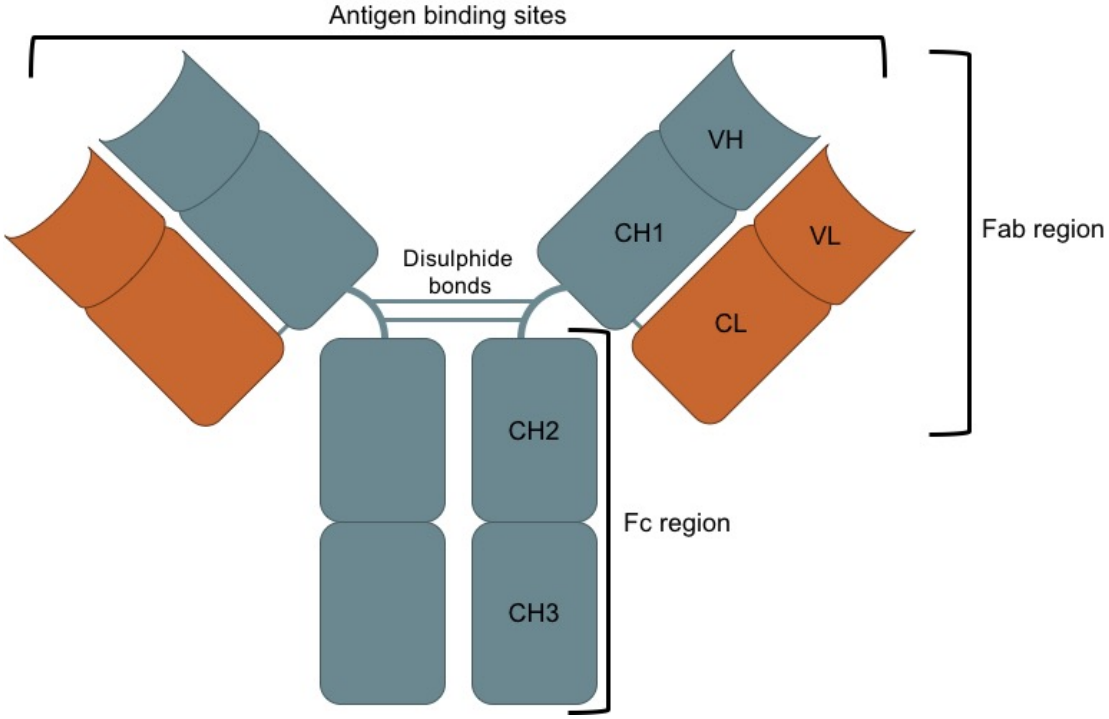

Figure 1.10 Structure of an antibody. Each antibody contains heavy (H) chains and light (L) chains, which are linked together by disulphide bonds. Each chain contains a variable region and a heavy chain.

1.4.2.1 Cell mediated Immunity

The function of T cells is in cell mediated immune responses. The TCR, although related to immunoglobulin, is very different. It is specifically adapted to detect antigens from pathogens (or non-self proteins) that have entered the host cells. In order for cell-mediated responses to occur, the T cell must directly interact with cells bound to the antigen which it will recognise. Cytotoxic T cells recognise any virus-infected cells, and kill the infected cell before viral replication is completed. The molecule called CD8 is mostly expressed on the surface of cytotoxic T cells. Th cells typically express the CD4 molecule on the cell-surface. Th cells, as mentioned before, have a special function in clearing infections. Th cells can be further divided into sub-groups based on the type of

pathogens they detect. For instance, the role of Th1 cells is in controlling intracellular bacterial infections. Th1 cells activate macrophages, and release cytokines such as IFN-γ, IL-2, and tumour necrosis factor (TNF)-β, which recruit more macrophages to the infection site [113,116,137]. Th2 cells control and support B cell activation and inhibit cell mediated immunity, by producing cytokines such as IL-4, IL-5, IL-10, and IL-13 [137]. As previously mentioned, Th17 cells have a distinct role in bacterial clearance by recruiting neutrophils and macrophages, and are the primary producers of the cytokine IL-17 [145–147]. T cells perform their functions by sensing peptide antigens from foreign proteins which have been displayed by molecules on the infected host cell surface. The molecules which display the peptide antigens are known as MHC molecules. There are two types of MHC molecule: MHC Class I and MHC Class II, which mostly share their major structural features, but differ in various smaller ways. MHC Class I are expressed on all cell types, with the exception of red blood cells. MHC Class II molecules are usually expressed on APCs (macrophages and DCs). Both classes of MHC molecule “trap” peptide antigens in clefts which are formed by two outer extracellular domains combining. Once the peptide is trapped, the MHC molecule can display it to T cells. Once a T cell containing a receptor specific for the peptide recognises the peptide, it can then interact with the APC. The key differences between MHC Class I and MHC Class II molecules is the type of peptides that they trap. MHC Class I molecules trap peptides originating from proteins synthesised in the cytosol, such as those from viruses [116]. MHC Class II molecules trap peptides originating from extracellular proteins (Figure 1.11). MHC Class I molecules are recognised by cytotoxic T cells, which kill the infected cell. Whereas MHC Class II molecules are recognised by specific effector Th cells [116]. The activation of the specific effector Th cells is supported by co-receptors that differentiate between the two classes of MHC molecule. As discussed above, the co-receptors include the CD8 co-receptor of cytotoxic T cells, which binds to MHC Class I molecules, and the CD4 co-receptors of Th cells, which bind to MHC Class II molecules. Upon target recognition, the relevant T cells are stimulated to release their specific set of effector molecules such as cytokines. The molecules the T cell releases will either directly influence the target cell or will recruit other effector cells [116].

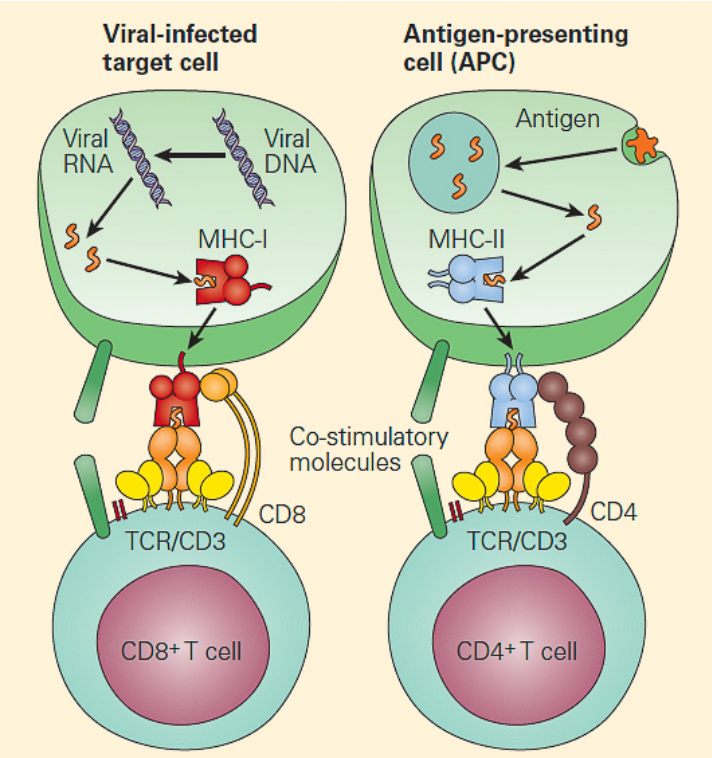

Figure 1.11 Presentation of antigens to CD8 and CD4 T cells from APCs via MHC complexes. Endogenous antigens are usually presented to CD8 T cells via MHC Class I molecules, whereas exogenous antigens are usually presented to CD4 T cells via MHC Class II molecules. Image taken from Stiehm R.E., 2012 [148].

1.4.3 KEY CYTOKINES AND TRANSCRIPTION FACTORS PRODUCED IN RESPONSE TO VIRAL AND BACTERIAL INFECTION

1.4.3.1 Interferons

Immune cells, such as macrophages, DCs, and NKs secrete large quantities of cytokines, including interferons (IFNs) [149–154]. The IFN family of antiviral cytokines are key during viral infections such as influenza. IFNs play a huge role in determining the outcome of influenza virus infection severity [155,156]. There are three types of IFNs, which are classified based on their amino acid sequence, and the type of receptor they signal through. The types are simply termed: type I IFNs, type II IFNs, and the newly described, type III IFNs. Type I IFNs include the subtypes IFN-α and IFN-β. In humans, there are thirteen distinct IFN-α proteins and a single IFN-β protein [157]. Type I IFNs all signal through a shared receptor; the IFNα/β receptor (IFNAR) and this signalling triggers downstream responses which provide anti-viral protection by inhibiting viral replication

[2]. Type I IFN induction is modulated by IRF family members [158–160]. IRF3 exists in the cytoplasm in an unactivated form, however it becomes phosphorylated during infection and contributes to early IFN-β production, which in turn triggers IRF7 synthesis. Production of IRF7 occurs through activation of the Janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) pathway, known collectively as JAK-STAT [161,162]. The IRF7 production leads to a subsequent increase in IFN-β expression, resulting in IFN-α production in a "positive amplification loop" (Figure 1.12) [163,164]. Type II IFNs (IFN-γ) are pro-inflammatory cytokines which modulate immune responses. IFN-γ is secreted from T cells and NK cells, and is involved in directing the adaptive immune response [114,116,165]. Type III IFNs were only very recently discovered, and are termed IFN-λ1, IFN-λ2, and IFN-λ3 [166]. Type III IFNs are not highly homologous to type I IFNs (15-20% homology), however their induction, signalling, and biological functions are very similar. Type I IFNs and type III IFNs are directly induced following virus infections and are the key IFNs secreted during influenza virus infection, both *in vivo* and *in vitro* [167,168].

To circumvent the anti-viral response elicited by the host, IAV has evolved numerous strategies such as inhibiting IFNs. The NS1 protein inhibits the host production of IFN and is generated by influenza in the early stages of IAV infection [169,170]. NS1 inhibits IFN by blocking the activation of transcription factors such as NF-κB and IRF3, which are essential in the production of type I IFNs [171,172]. This contributes to evasion of the host's innate immune responses.

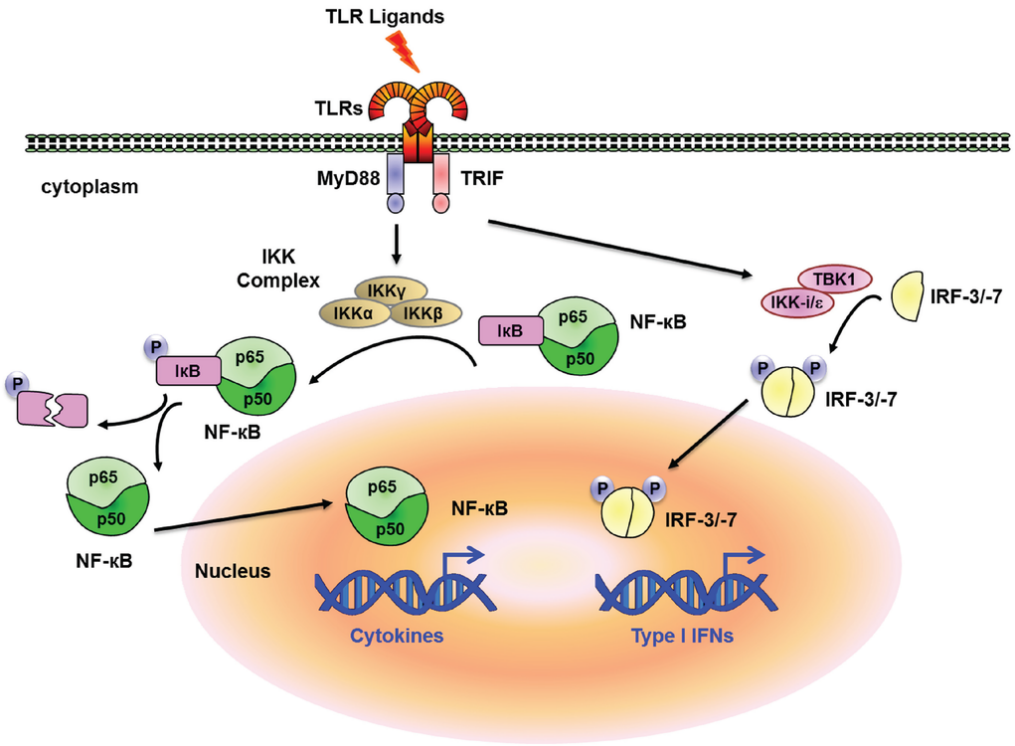

Figure 1.12 TLR signalling and NF-κB activation leading to downstream activation of type I IFNs through IRF3 and IRF7 signalling. Image taken from Tartey *et. al.*, 2015 [173].

1.4.3.2 The T helper 17 and T helper 1 Response in Bacterial Clearance

The Th17 response has been identified as critical in the effective clearance of *S.p.* from the lung [88,174,175]. Th17 cells are distinct from Th1 and Th2 cells, which control intracellular bacterial infections and parasitic infections respectively. Th17 cells are the primary producers of interleukin-17 (IL-17, also known as IL-17A), a pro-inflammatory cytokine, with a specific role in the recruitment of neutrophils and macrophages [145–147]. It is well known that the cytokines, transforming growth factor-β (TGF-β), IL-6 and IL-1β drive Th17 differentiation, while IL-23 conserves the expansion and commitment to the Th17 lineage, thus increasing the production of IL-17A [176–181]. IL-27 has been shown to function both as an inducer of Th17 cells and an inhibitor of Th17 cells [182]. The anti-inflammatory cytokine, IL-10 inhibits production of IL-12 and Th1 differentiation, and has also been shown to inhibit Th17 responses [183,184]. IL-12p70 is a pro-inflammatory cytokine, which promotes Th1 differentiation through the production of IFN-γ, both of which are important in the response to microbial pathogens [185].

1.4.3.2.1 Interleukin-23

Interleukin-23 (IL-23) is a member of the IL-12 cytokine family, and is important for the expansion and maintenance of Th17 cells. IL-23 is comprised of an IL-23p19 subunit and an IL-12p40 subunit which also serves as a subunit for IL-12 [186]. IL-23 signals through IL-23R and IL-12Rβ1, stimulates signalling through the JAK-STAT pathway, and phosphorylates the transcription factor, STAT3 [187]. In mice, TGF-β and IL-6/IL-21 induce the surface expression of IL-23R on Th17 cells, which IL-23 subsequently interacts with [177,188,189]. Exposure to these cytokines induces expression of retinoic acid receptor (RAR)-related orphan receptor-γt (RORγt), and thus, encourages expression of IL-23R and IL-17 in mice [190]. IL-23R signalling stimulates phosphorylation of STAT3 in humans, which induces transcription of IL-23R and RAR-related orphan receptor C (RORC), which encodes RORγ. This creates a positive feedback loop, and leads to controlled expression of genes which control the activation of T cells [187,191–193]. T cell activation in the presence of IL-23 promotes the expansion of Th17 cells in mice [194].

1.4.3.2.2 RAR-related orphan receptor C (RORC)

RAR-related orphan receptor C (RORC) is the gene in humans which encodes for two protein isoforms: RAR-related orphan receptor gamma (RORγ) and RORγt, previously known as RORC1 and RORC2, respectively [195]. RORγt, which is exclusively expressed in the thymus, is a transcription factor, which has been identified as a potential master regulator for driving Th17 cell differentiation in both mice and humans [190,196–201]. Expression of RORγt is induced by TGF-β and IL-6 or IL-21 in a STAT3-dependent manner in humans and mice [190,202–204], which is key for the expression of IL-17 [190]. In mice, an additional transcription factor, RORα has also been implicated in Th17 differentiation [205], however the influence of RORα in humans appears to be weaker [206]. As mentioned above, RORγt also plays an important role in the upregulation of IL-23 [190]. Interestingly, in mice and humans, IL-27 has been shown to inhibit RORC expression in a STAT1-dependent manner, which resulted in IL-17A and IL-17F inhibition [207].

1.4.3.2.3 Transforming growth factor-β (TGF-β)

Transforming growth factor-β (TGF-β) is a multifunctional cytokine, belonging to the transforming growth factor family of cytokines. It has both pro-inflammatory and anti-inflammatory capabilities. It acts as an immune regulator, and regulates functions ranging from tumour cell recognition, to suppression of autoimmune responses. TGF-β can regulate T cells to maintain homeostasis [208]. In humans, TGF-β is essential for the differentiation of Th17 cells. TGF-β can synergise with either IL-21 or IL-6 and IL-23, to induce RORC, which is the human version of RORγt in mice [204,209]. Human naïve T cells must be exposed to TGF-β in order to express IL-23R [204,209,210]. TGF-β is secreted by many cell types including macrophages and T_{regs} [211]. Although, essential for differentiation of Th17 cells, once the infection has been cleared, TGF-β negatively regulates Th17 responses to maintain homeostasis [208]. When TGF-β is synergised with IL-6, the Th17 response is induced, however, when TGF-β is secreted alone, the Th17 response is inhibited [176].

1.4.3.2.4 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is predominantly a pro-inflammatory cytokine, part of the IL-6 family of cytokines. The biological functions of IL-6 are mediated by the receptor complex, which consists of the IL-6 binding type I transmembrane glycoprotein, known as IL-6R, and the type I transmembrane signal transducer protein gp130. Initially, IL-6 binds to the membrane-bound non-signalling α-receptor IL-6R (mbIL-6R). The IL-6/IL-6R complex subsequently binds to two molecules of gp130, resulting in IL-6 signal transduction through JAK/STAT activation [212]. Macrophages, neutrophils, and certain T cells express IL-6R on the cell surface, however gp130 is universally expressed [213]. IL-6 is critical during the transition from innate immunity to adaptive immunity. IL-6 is key in the initial attraction of neutrophils immediately after detection of microbial products, however after 24-48 hours, the infiltration of neutrophils is replaced by macrophages, DCs and T cells to prevent tissue damage. This occurs due to proteolytic processing of IL-6R, which causes a change from neutrophil recruitment to monocyte recruitment. The change occurs by inhibiting neutrophil-attracting chemokines such as CXCL1, and inducing monocyte-attracting chemokines such as CXCL5 [214–216]. Additionally, IL-6 has a role in preventing T cells from apoptosis, which relies on STAT3 [217,218]. IL-6 is also fundamental in the differentiation of B and T cells. IL-6 enhances B cell helper

functionality of $CD4^+$ T cell by induction of IL-21 [219,220]. IL-6 can skew T cell differentiation towards Th17 responses by synergising with TGF-β, which inhibits TGF-β-mediated differentiation of naïve T cells into T_{reg} cells, and thus induces Th17 cells [176,177]. TGF-β increases IL-6 induced STAT3, which enhances expression of RORγt, leading to Th17 differentiation [221]. The combination of IL-6 and IL-1β is necessary for the enhancement of the Th17 lineage, but these cytokines together have also been implicated in the pathogenic effects of Th17 cells [191,204]. In such instances, IL-6 upregulated IL-23R in naïve $CD4^+$ T cells via STAT3 [191]. It has been demonstrated that the differences between differentiation of Th17 cells and the maintenance of Th17 cells is due to the alternative IL-6 signalling pathways. Th17 differentiation is mediated by classic signalling of IL-6 (via the membrane-bound IL-6R), whereas Th17 maintenance is mediated by "trans"-signalling of IL-6 (via a soluble IL-6R) [222].

1.4.3.2.5 Interleukin-1β (IL-1β)

Interleukin-1β (IL-1β) is a strong pro-inflammatory cytokine and a member of the IL-1 family of cytokines. It is key in host responses to infection, however the margin between host defence and damage to the host is very narrow [223]. IL-1β is produced and secreted by many different cell types such as monocytes and macrophages [224]. Initially, an inactive precursor called pro-IL-1β is produced in response to PAMP recognition through PRRs on macrophages [225]. The production of pro-IL-1β is a priming step and a further PAMP must be recognised before the active IL-1β molecule can be secreted [224]. When pro-IL-1β is cleaved by the pro-inflammatory protease, caspase-1, active IL-1β is rapidly secreted [226,227]. During extracellular bacterial infections, IL-1β is produced, and it induces and amplifies IL-17A production from Th17 cells [178].

1.4.3.2.6 Interleukin-27 (IL-27)

Interleukin-27 (IL-27) is a member of the IL-12 family of cytokines, which initially was shown to contribute to Th1 immunity [228]. However, later studies have shown that IL-27 has the ability to supress Th1, Th2, and Th17 responses [229–233]. The contradictory effects of IL-27 on Th17 responses may, in part, be due to the transcription factors, STAT1 and STAT3. STAT1 is known to be a strong inhibiter of Th17 responses, whereas

STAT3 is known to be a strong inducer of Th17 responses [203,233–236]. IL-6 induces both STAT1 and STAT3 strongly. IL-27 also induces both STAT1 and STAT3, and it has been suggested that it may be the ratio of phosphorylated STAT1/STAT3 that dictates whether IL-27 will exert pro- or anti-Th17 effects [182]. IL-27 functions as a heterodimer comprising p28 and EBV-induced gene 3 (Ebi3), which are homologous to IL-12p35, and p40, respectively [228,237]. The IL-27R complex consists of the gp130 molecule of IL-6R, and a unique subunit IL-27R. The IL-27R complex as stated above, activates STAT1 and STAT3 via phosphorylation, which is mediated by the JAK pathway [238,239]. IL-27 inhibits Th17 differentiation by blocking up-regulation of RORγt in naïve T cells in a STAT1 dependent manner in mice [207]. Although, IL-27 can prevent Th17 differentiation, it does not affect already committed Th17 cells. However, IL-27 also stimulates T cell production of anti-inflammatory, IL-10, which can directly control Th17 cell production [238,240,241].

1.4.3.2.7 Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is an anti-inflammatory cytokine, which is broadly expressed [242]. T_{reg} cells are the main producers of IL-10, and IL-10 is typically considered to be a cytokine with inhibitory effects on Th17 responses which protect against the pathogenic effects associated with Th17 cell production [211,243,244]. However, studies have shown that Th17 cells can also produce IL-10 [245,246]. IL-10 has been shown to reduce lung pathology of severe influenza infection in mouse models [247]. Additionally, IL-10 has also been shown to enhance the susceptibility to secondary bacterial infections following influenza infection in mice [89]. IL-10 can control Th1 and Th17 immune responses in mice [241,246,248,249]. IL-27 can strongly induce production of IL-10 by immune cells, and type I IFN can induce production of IL-10 during infection [207,247]. IL-10 is expressed by both innate and adaptive cells such as DCs, macrophages, NK cells, and as mentioned before, Th17, and T_{reg} cells [183]. The anti-inflammatory effects of IL-10 are mediated through the IL-10 receptor (IL-10R), which is composed of two subunits (IL-10R1 and IL-10R2). Interaction between IL-10 and IL-10R activates the JAK/STAT pathway. IL-10 induces phosphorylation of STAT3 and STAT1 [183,250–252].

1.4.3.2.8 Interleukin-12p70 (IL-12p70)

Interleukin-12 (IL-12) is a member of the IL-12 family of cytokines and is composed of two chains; p40 and p35 [253,254], of which the former also serves as a subunit for IL-23 [186]. The IL-12 receptor (IL-12R) also comprises of two chains IL-12Rβ1 and IL-12Rβ2, signalling through which activates the JAK-STAT pathway [255]. During microbial infection, DCs and macrophages are the main producers of IL-12 [256]. IL-12p40 is produced in considerable excess over the p35 subunit of IL-12 and IL-23 [253]. IL-12 is a strong inducer of IFN-γ from T cells and plays an important role in inducing Th1 differentiation [257]. In turn, IFN-γ also has the ability to enhance IL-12 production, which forms a positive-feedback loop during Th1 responses [254]. The pro-inflammatory effects of IL-12 are often mediated by IFN-γ. Although, IL-12p70 is a Th1 cytokine, it has been shown to be triggered by *S.p*. [258–260].

1.4.3.2.9 Interleukin-17A (IL-17A)

Interleukin-17 A (IL-17A) is part of the IL-17 family of cytokines. The family consists of the cytokines IL-17A-IL-17F, and all have a role in inflammatory responses. IL-17A is produced by immune cells such as Th17 cells and γδT cells, and plays a crucial role in host defence against microbial infections [147,261–264]. The IL-17A receptor (IL-17RA) is a type I transmembrane protein, which is ubiquitously expressed [265]. Signalling through this receptor activates downstream signalling pathways and leads to the production of pro-inflammatory cytokines such as IL-6 [266]. Studies have shown that IL-17A is not sufficient to mediate IL-17A signalling, and IL-17RA must heterodimerise with IL-17RC in order for IL-17A to signal through it [267]. IL-17A activates NF-κB, which is dependent on TRAF6 and TAK1 [268]. IL-17A can generate signalling cascades which lead to neutrophil recruitment, inflammation, and host defence. However, IL-17A can also be damaging to the host through excessive inflammation, mostly by synergising with other cytokines [264,269]. High levels of IL-17A have been linked with development of autoimmune inflammatory conditions such as multiple sclerosis (MS), psoriasis and asthma, amongst others [270–272]. IL-17A can be negatively regulated by TRAF4 and TRAF3 through competitive binding [273,274]. As mentioned above, IL-17A is important in host defence against extracellular bacterial pathogens, functioning via neutrophil recruitment [147]. IL-17A is crucial in the clearance of *S.p.* [175]. There are subtle differences between mouse and human with regards to the pathway leading to IL-17

production, for instance with divergence reported in the stimulations required to induce RORγt. In mice, TGF-β and IL-6 induce RORγt, however there is contradictory evidence regarding the human pathway [190]. Some studies in humans have found that TGF-β and IL-21 are more successful in inducing RORC [204], whereas others have corroborated results found in mouse models [209,210,275].

1.4.3.2.10 Interferon-γ (IFN-γ)

Interferon-γ (IFN-γ), also known as type II IFN, is a potent pro-inflammatory cytokine, which is produced by many cells including Th1 cells. It is also produced by macrophages, DCs, NKs and antigen-activated T cells [276–278]. IFN-γ production is regulated by IL-12p70 [257]. IL-12p70 is a very efficient inducer of IFN-γ, even at low concentrations [279]. In T cells, IL-12 synergises with IL-2 and stimulates the TCR-CD3 complex, resulting in activation of the CD28 receptor and rapid production of IFN-γ [254]. In T cells, there are two distinct pathways which induce IFN-γ; one is induced via receptors such as TCRs, and CD3, the other is induced by IL-12 in combination with IL-18 [280–283]. In turn, IFN-γ also induces expression of IL-12Rβ2 by T cells [284], resulting in a positive feedback loop. IFN-γ has many roles including activation of phagocytes and stimulation of antigen presentation through MHC Class I and II expression [285,286]. IFN-γ is key in defence against microbial pathogens [287]. However, there is contradictory evidence concerning the role of IFN-γ in *S.p.* infections, which are detailed below.

1.4.3.3 The Th17 Response and Type I and Type II Interferons in Streptococcus pneumoniae infection

Viral induction of type I and type II interferons, which are strongly produced in the late response to IAV infection have been shown to inhibit Th17 responses in mice [2,3]. A study in mice found that without functional IFNAR signalling, mice were more resistant to secondary bacterial pneumonia and superinfection post-influenza than mice with functional IFNAR signalling [2]. However, in contrast to these reports, it has been shown that IFN-α expression prior to respiratory infection with *S.p.* improved the outcome of pneumococcal infection in mice, and that IFNAR signalling can be crucial for *S.p.* bacterial clearance in mice [288–292]. There are also conflicting reports as to the effects of type II IFN (IFN-γ) during *S.p.* infection in mice. It has been shown that IFN-γ did not

have a protective role against *S.p.*, as IFN-γ receptor-deficient (IFN-γR$^{-/-}$) mice were found to have significantly fewer pneumococci in their lungs than wild-type mice, and IFN-γ$^{-/-}$ mice had fewer colony-forming units present in the lungs than wild-type mice [293]. Another study found that in the presence of an influenza infection, IFN-γ produced in the lung by T-cells inhibited macrophage-mediated bacterial clearance, which is essential in the clearance of pneumococci, resulting in an increase in susceptibility to secondary bacterial infections [3]. In contrast to these studies, it has been shown that IL-12 and IFN-γ can mediate protective effects against *S.p.* by promoting neutrophil accumulation [185]. Additionally, IFN-γ produced by neutrophils during *S.p.* infection was important in host defence as mice deficient in IFN-γ had impaired bacterial clearance [294]. There have been a significant number of studies carried out in animal models, however there is a scarcity of studies in human models. Due to this lack of research in human models coupled with the discrepancies in the effect of IFNs on Th17 responses, more research must be carried out so that this complex relationship can be fully explored.

1.4.4 THE TH17 RESPONSE AND INFLAMMATION

A properly functioning immune system is necessary, not just for controlling infections, but for maintaining the balance of “self” and “non-self” recognition. Often the immune system can over-compensate, meaning a “normal” immune response will be mounted, but will be directed at non-infectious antigens, as is the case with allergic reactions. The same issue occurs in autoimmune diseases, where the immune system mounts a response incorrectly against a “self” antigen. Or in transplant rejection, where the body is correctly recognising the transplant tissue as “non-self”, but the correct responses would be detrimental to the host, and not beneficial [113,116]. As mentioned above, the Th17 response is vital in the clearance of bacterial infections from the lung due to the production of the cytokine, IL-17 [88,174,175]. However, IL-17 has also been shown to be responsible for the development of inflammation in a range of autoimmune diseases such as rheumatoid arthritis (RA), psoriasis, and Crohn’s disease [198,295–297]. As there is a balance to be struck between the benefits of Th17 responses and harmful effects of it, future treatments targeting the Th17 pathway (either in an agonistic or antagonistic fashion) should be carefully considered.

1.4.5 OVERVIEW OF NORMAL IMMUNE RESPONSES TO INFLUENZA AND STREPTOCOCCUS PNEUMONIAE

As with other pathogens, the innate immune response acts as the first line of defence against both IAV and *S.p*. As mentioned before, PAMPs from these pathogens are recognised by PRRs which trigger activation of the innate immune system, resulting in the production of cytokines and anti-viral and anti-microbial molecules [96,298–300].

1.4.5.1 The Normal Immune Response to Influenza A Virus

Once PAMPs from IAV are recognised by innate PRRs, transcription factors such as IRF3 and IRF7 are activated, which trigger anti-viral interferon and cytokine production [115,133]. Both T cells and B cells are important in the adaptive immune response to IAV infection. With the help of type I IFNs, CD8 T cells differentiate into cytotoxic T cells, which kill IAV-infected cells [298,301–303]. CD4 T cells also contribute to adaptive immunity against IAV by aiding B cell activation and production of antibodies [304]. CD4 T cells can also differentiate into Th1 cells, which secrete anti-viral cytokines such as IFN-γ [305]. B cells generate antibodies which enable viral destruction [306].

1.4.5.2 The Normal Immune Response to Streptococcus pneumoniae

PRRs such as TLRs recognise *S.p.* infection and once activated, they regulate the production of pro-inflammatory cytokines. The pro-inflammatory cytokines, in turn, recruit neutrophils and macrophages and initiate adaptive immunity [96,307]. In response to *S.p.* infection, CD4 T cells differentiate into Th17 cells, which recruit neutrophils and macrophages to the site of infection by the production of IL-17 [145–147,308]. In addition to T cells, B cells also play a role in the adaptive response to *S.p*. Once naïve B cells are stimulated by polysaccharides from *S.p*., they can secrete the *S.p.* specific IgA antibody, which opsonises *S.p*., and encourages phagocytosis [309,310].

1.4.5.3 The Immune Response to Influenza and Streptococcus pneumoniae Co-Infections

Both IAV and *S.p.* are significant respiratory pathogens which cause considerable damage to public health [18,93,94]. Individually, each pathogen can be highly

pathogenic, however when these pathogens co-occur, the damage caused by this synergy is often far more critical. There have been many hypotheses as to why this occurs, such as damage to the epithelium, and cytokine storms. However, more recently, impaired immune responses have been implicated [57,311–315].

1.5 MODELS FOR INFLUENZA VIRUS INFECTION

Many studies performed in this field are performed in animal models, particularly mice [102,316–319]. Although these models provide useful insights into *in vivo* responses, the research can often be contradictory [102,316], with the relevance of mouse models being called into question [320,321]. A study which compared immune responses of laboratory mice to those of wild mice found striking differences between them and questioned not only the relevance of using laboratory mice to model human responses, but also to model wild mice responses [322]. Additionally, knock-down or gene-deficient mice are often used, however the results obtained from these studies must be reported with care due to the complex nature of immune responses. As immune responses to pathogens often cause collateral damage (such as an increase in neutrophils during viral infection), knock-down models can be vulnerable to mis-interpretation of causal relationships [323]. Many have suggested that ferrets are the appropriate animal to use to model influenza and bacterial co-infections as they are susceptible to infection with human influenza viruses, whereas mouse models require prior host adaptation of the human viruses [324,325]. Additionally, ferrets can transmit the virus to other non-infected ferrets, and they have a high proportion of α(2,6) linkages, which are the linkages preferred by human adapted viruses [326]. However, there are limitations to these models. Due to the size of the animal, their associated housing requirements are higher than other smaller animals, which in turn increases the cost of these studies. This results in small group sizes, which mean that any statistical analyses performed are very limited [327]. Although, animal models have paralleled certain human immune responses, research should also be performed in human models to help corroborate such studies.

For many years, the standard *in vitro* assays have been to culture DCs in the presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) to represent the monocyte derived DC [328–331]. However, the relevance of these conventional DCs *in vivo* is unclear as stimulations involving IL-4 is unlikely to characterise the cytokine environment present at the site of a viral infection [332,333]. As type I IFN are produced in response to viral infections [334,335], many studies have cultured DCs in the presence of type I IFN (IFN-DC) [332,336,337]. However, IFN-DCs have extra characteristics which mark them as distinct from DCs *in vivo* [336,338,339]. Due to these issues, our lab group sought to develop a human immune model which would more closely reflect the *in vivo* human response to viral infections.

1.5.1 EX VIVO HUMAN IMMUNE MODEL

Our lab group have developed an *ex vivo* human immune model (Figure 1.13), which has been used in published work, based on the isolation of primary human immune cells and purification of $CD14^+$ monocytes which are directly infected/stimulated, without pre-priming of the immune response [333,340,341]. In a previous study, our lab group comprehensively compared the maturation profiles (phenotypic expression, cytokine production, apoptosis, and T cell proliferation) of directly infected-untreated monocytes, IL-4 treated DCs, IFN-treated DCs, and DCs following co-culture with supernatant from influenza infected lung epithelial cells. The different cells and treatments were chosen as they are all commonly used to model what happens at the site of infection, however their suitability has been questionable. That study found that direct influenza infection of monocytes and monocytes cultured with supernatants from influenza infected lung epithelial cells induce distinct DC subsets compared with influenza infection of artificially generated IL-4-treated DCs and IFN-treated DCs. Additionally, differences also occurred in T cell responses to each DC subset, showing that the model used for generating DCs is critical in the outcome of influenza infection as different DCs may respond differently to the same virus. This can create conflicting data. It was concluded that artificially generated DCs (such as those treated with IL-4 and IFN) skewed immune responses to influenza infection, whereas direct influenza infection of monocytes mimic those generated from supernatants from influenza infected epithelial cells and appear to more accurately mimic those generated *in vivo*. This model is based on comprehensive analysis of the marker signals produced by influenza infected lung epithelial cells which will influence the extravasation and maturation of monocytes from the periphery [333]. The purified $CD14^+$ monocytes act as antigen presenting cells (APCs) upon stimulation and as the link between the innate and adaptive immune responses. Thus, in this study we chose to infect primary human monocytes isolated from healthy volunteers directly with influenza and assess the impact these infections have on innate responses to different stimulants. Going forward isolated $CD14^+$ monocytes, will be referred to as APCs. To ascertain the adaptive responses, a mixed lymphocyte reaction (MLR) was used, which is commonly used in the investigation of human T cell responses [333,342–347]. In this assay, as per previous studies [333,342], infected/treated APCs from one donor were co-cultured with human primary T cells from a different donor resulting in an allogeneic response due to the mismatched MHC antigens. This model uses allogeneic T cells as opposed to an antigen-specific response assay as there is likely to be too few autologous T cells recognising IAV and *S.p.* in circulation in the periphery of healthy donors, which would be insufficient to perform a whole study across a variety of

treatments. By using allogeneic T cells, a sufficiently strong signal is produced to mount responses for analysis (personal communication: Dr Johnson).

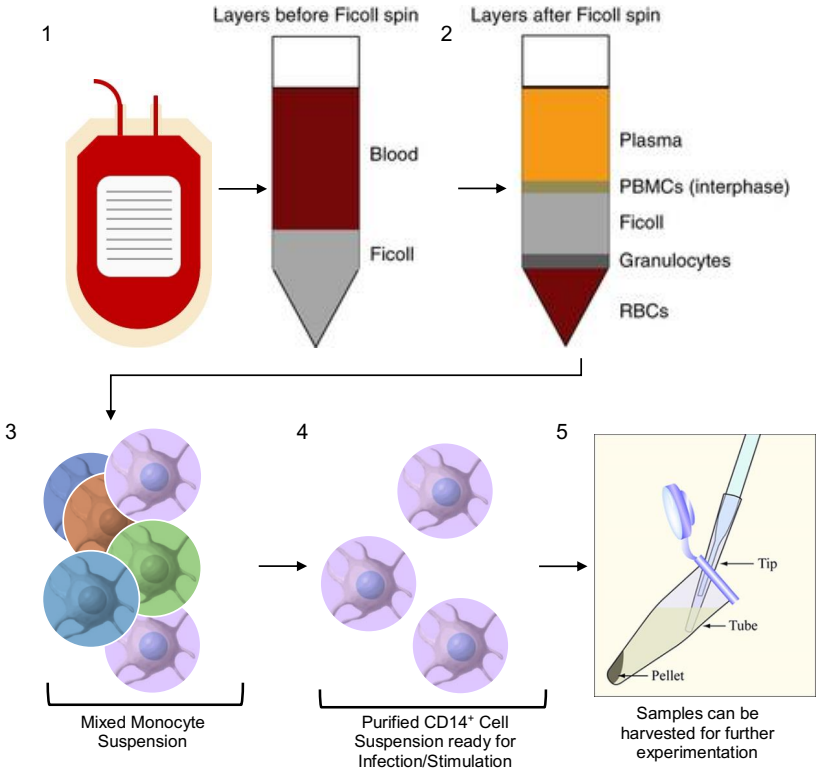

Figure 1.13 Scheme of Human Immune Model. Step 1: Blood donations are obtained from healthy human donors from the Irish Blood Transfusion Service. Step 2: Whole blood is separated into distinct components using gradient centrifugation [348]. Step 3: Peripheral blood mononuclear cells (PBMCs) are removed which contains a suspension of mixed monocytes. Step 4: $CD14^+$ monocytes are separated from PBMCs using magnetic microbead separation and can be used for infections or stimulations. Step 5: $CD14^+$ monocyte suspension can be harvested for downstream applications.

1.6 OBJECTIVES OF STUDY

Influenza A Virus and *Streptococcus pneumoniae* are both major pathogens which pose substantial public health problems [18,93,94]. Although each pathogen can individually cause serious infections, when these pathogens synergise, the combined effect often becomes overwhelming for the immune system, leading to critical complications. Influenza-*S.p.* co-infection can result in hospitalisation and/or death of both patients with pre-existing lung disease or previously healthy individuals [4,5]. The mechanisms behind this synergy are still not understood, which has prevented the development of appropriate therapeutic treatments.

The overall aim is to further understand the mechanisms that trigger susceptibility to bacterial disease during IAV infection. We sought to investigate if IAV infection can alter appropriate innate and adaptive immunity to *S.p.* in humans and whether this may lead to an increase in anti-inflammatory cytokines. Subsequently, we sought to investigate whether observed inhibition of *S.p.* responses was dependent or independent of type I IFN responses. The next objective was to investigate if IAV infection targets specific TLRs involved in sensing *S.p.* infection, and whether TLR5 agonism can restore inhibited responses to *S.p.* With a view to determining possible targets for IAV to mediate inhibition, the Th17 response transcription factor, RORC was examined. Additionally, the effect of the IAV component, HA, is of great interest as it is used as in vaccines. We aim to determine the effect of HA on *S.p.* responses and on responses to TLR agonists.

Objectives:

- Establish immune responses to new clinical isolates and batches of influenza and *S.p.* and further characterise the model,
- Confirm or rule out the involvement of type I IFN by extensive examination of mRNA and protein, via quantitative polymerase chain reaction (qPCR) and ELISA, respectively,
- Identify responses to individual *S.p.*-associated TLR agonists in humans (*ex vivo* human immune model),
- Establish if influenza selectively targets *S.p.*-associated TLR signalling in humans,
- Ascertain whether TLR5 agonism may be useful in circumventing IAV-inhibition of *S.p.* responses and,
- Examine effect of HA on *S.p.* responses and responses to TLR agonism.

2.0 FURTHER DEVELOPMENT OF HUMAN *EX VIVO* MODEL AND CHARACTERISATION OF NEW VIRAL AND BACTERIAL STOCKS

2.1 INTRODUCTION

One of the most prevalent respiratory viral pathogens is influenza. Current estimates state that approximately half a million deaths are caused by seasonal influenza virus annually [30,31]. Most of these deaths are due to secondary bacterial pneumonia [18,85]. The secondary bacterial infections are typically caused by capsular, extracellular bacteria such as *S.p.* [1–3]. Initially, Th1 cells were thought to be the crucial cell group in clearing bacterial infections [266], however, recently Th17 cells have been identified as critical in the effective clearance of *S.p.* from the lung [88] and further studies have revealed that IAV infection has been shown to inhibit the Th17 response in mice [2,349]. A large proportion of the research carried out in this field is performed in animal models, particularly mice with relatively few studies performed in human models [102,316–319]. Much of the research that is performed in human models use pre-treated DCs, however such treatments have been shown to skew immune responses [333]. Due to this, our lab group developed an *ex vivo* human immune model with which to examine viral infections such as IAV. Additionally, the model was developed with the intention of utilising it to distinguish if certain strains of IAV may be more likely to lead to a predisposition to secondary bacterial infections. The human *ex vivo* model was previously used to examine the effect of IAV infection on responses to *S.p*. That study used both a lab-adapted H1N1 virus (H1N1/A/PR8/24) and clinical isolates of IAV (H3N2/A/Wisconsin/67/2005 or H3N2/A/Panama/2007/99) along with in-house generated heat killed *S.p.* It demonstrated that IAV inhibited important pneumococcus driven innate cytokines in human APCs and adaptive cytokines in T cells [350]. However, for subsequent studies, a new batch of the lab-adapted H1N1 virus and a new clinical isolate of H3N2 (H3N2/A/Uruguay/716/2007) were obtained from the National Institute for Biological Standards and Controls (NIBSC). Additionally, new batches of commercially bought *S.p.* (Invivogen) were used instead of in-house generated *S.p.* for this study. Due to known differences in immune responses to different IAV strains and bacterial subtypes, any further studies using new batches of IAV and *S.p.* needed to be further characterised using the human immune model [302,309,351].

To confirm the ability of IAV to infect APCs, an assay was developed to detect for the presence of the IAV nucleoprotein, which is essential for viral replication and is intracellularly expressed during influenza infection [340,352].

To assess the impact on innate APC responses that direct Th17/Th1/T_{reg} responses, secreted cytokines (IL-23, IL-6, IL-1β, TGF-β, IL-27, IL-12p70, and IL-10) were measured in the supernatants of untreated cells or cells stimulated with heat inactivated *S.p.*, infected with live H1N1 or H3N2 alone or in combination with *S.p*. exposure. IL-23, IL-6, IL-1β, and TGF-β are essential in Th17 responses to *S.p*. Although, TGF-β induces the Th17 response, it is also an anti-inflammatory cytokine which, in the absence of IL-6, can inhibit the pro-inflammatory Th17 response [176,353]. Interleukin-27 (IL-27) is of interest as it was initially shown to contribute to Th1 immunity [228,354], although later studies have shown that it may also be involved in Th17 differentiation [182]. IL-12p70 is involved in the Th1 response through the production of type II interferon (IFN-γ) [257,355] and IL-10 induces T_{regs} and has been shown to inhibit Th1 and Th17 responses [241,246,248,249,356].

Innate immune cells can prime adaptive immune cells such as Th cells to induce long-term immunity [116,137]. In a normal immune response, when a pathogen is sensed by the immune system, APCs present antigens to naïve T cells via their MHC class II molecules. The antigen is recognised by the naïve T cell, which leads to T cell clonal expansion and differentiation into effector T cells such as Th17/Th1 or T_{reg} cells, depending on which innate cytokines are produced [116,137,138]. The innate cytokines have a profound effect on the adaptive response which is subsequently elicited [116,137,138].

To examine the adaptive immune responses to new clinical isolates of live IAV and *S.p.* infection, a mixed lymphocyte reaction (MLR) was used. This reaction is commonly used to investigate T cell responses [116,333,342–347,357–359]. In this assay, infected/treated APCs from one donor were co-cultured with T cells from a different donor, resulting in an allogeneic response due to mis-matched MHC antigens. To assess the impact on adaptive T cell responses that direct Th17/Th1/T_{reg} responses, secreted cytokines (IL-17A, IFN-γ, TGF-β, and IL-10) were measured in the supernatants of untreated cells or cells stimulated with heat inactivated *S.p.*, live H1N1 or H3N2 alone or in combination with *S.p*., H1N1 HA or H3N2 HA alone or in combination with *S.p*.

Th17 cells are the primary producers of IL-17A, which has a specific role in the recruitment of neutrophils and macrophages [145–147]. IL-17A plays a crucial role in host defence against microbial infections and is crucial in the clearance of *S.p*. [147,175,261–264]. IFN-γ is a potent pro-inflammatory cytokine, which is produced as

part of the Th1 response by antigen-activated T cells, macrophages, and DCs [276–278]. IFN-γ production is regulated by IL-12p70 [257]. IL-12p70 is a very efficient inducer of IFN-γ, even at low concentrations [279]. IFN-γ has many roles including activation of phagocytes and stimulation of antigen presentation through MHC Class I and II expression [285,286]. IFN-γ is key in defence against microbial pathogens [287]. However, there is contradictory evidence concerning the role of IFN-γ in *S.p.* infections.

Specific aims of this chapter were to:

- Confirm ability of new batch of H1N1 and new clinical isolate of H3N2 to infect APCs,
- Determine the variability of infectivity of influenza from donor to donor,
- Establish innate and adaptive immune responses to a new bank of influenza strains and HKSP,
- Investigate the effect of new influenza strains on responses to HKSP,
- Determine role of anti-inflammatory cytokines on HKSP and IAV co-infections and,
- Establish the levels of apoptosis in HKSP-treated and IAV-infected human immune cells using flow cytometry.

2.2 MATERIALS AND METHODS

Table 2.1 List of reagents used in this study

Product	Catalogue Number	Company
Anti-beta Actin antibody	ab8227	Abcam, UK
Prism Ultra Protein Ladder	ab116027	
Goat anti-rabbit IgG H&L (HRP)	ab6721	
FITC Annexin V Apoptosis Detection Kit I	556547	BD Becton Dickson, UK
FACSFlow Sheath Fluid	342003	
FACS Clean Solution	340345	
FACSRinse Solution	340346	
5 ml Polystyrene Round-Bottom Tube	362054	
Sodium pyruvate	11360-039	Bio-sciences Ltd., Dun Laoghaire, Ireland
RPMI 1640 Medium GlutaMAX Supplement	61870-101	
Lymphoprep	1114545	
Hanks Balanced Salt Solution (HBSS)	24020091	
Invitrogen Human IL-23 ELISA kit	88-7237-88	
Invitrogen IL-12p70 Human ELISA kit, High Sensitivity	BMS2E8HS	
Penicillin Streptomycin (5,000 U/ml)	15070063	
Clear Flat-Bottom Immuno Non-sterile 96-well maxi-sorp plate	442404	
1X TMB solution	00-4201-56	
ThermoScientific Reagent Reservoirs	95128093	
Sealing Tape for 96-well plate	15036	
Dulbecco's phosphate buffered saline (10X)	14000059	
RPMI 1640 Medium GlutaMAX Supplement, HEPES	72400021	
Nuclease-Free Water (Not DEPC-treated)	AM9938	
RNaseZAP Rnase Decontamination Solution (250 ml)	AM9780	
SuperSignal™ West Dura Extended Duration Substrate	37075	
Invitrolon™ PVDF/Filter Paper Sandwich	LC2005	
Pierce BCA Protein Assay Kit	23225	
Bolt™ 4-12% Bis-Tris Plus Gels, 12-well	NW04122BOX	
20X Bolt™ MES SDS Running Buffer	B0002	
4X Bolt™ LDS SDS Sample Buffer	B0007	

10X Bolt™ Sample Reducing Agent	B0009	
Foetal calf serum (FCS)	S1830-500	Bio-sera, France
H1N1 NP Primers	N/A	Eurofins Genetic Services, UK
H3N2 NP Primers		
Ethidium Bromide (Eb)	10714181	Fisher Scientific, Dublin, Ireland
Sodium Chloride (NaCl), Extra Pure	10112640	
Sodium Chloride (NaCl)	10735921	
Glass cloverslip No. 1 (22 mm x 22 mm)	12333128	
Bovine serum albumin (BSA) protease-free powder	1287-1630	
1X Phosphate Buffered Saline (PBS), pH 7.4	10173433	
Water, Nuclease-free, Molecular Biology Grade	15835408	
RIPA Lysis and Extraction Buffer 100mL	10017003	
Halt Protease and Phosphate Inhibitor Cocktail (100X)	10085973	
Sulphuric acid (H_2SO_4)	84721	Fluka, Germany
Heat Killed *Streptococcus pneumoniae* (*HKSP*)	tlrl-hksp	Invivogen, Toulouse, France
Potassium dihydrogen phosphate (KH_2PO_4)	1.04871.1000	Merck, Damstadt, Germany
$CD14^+$ microbeads	130-050-201	Miltenyi Biotech, Surrey, UK
$CD3^+$ microbeads	130-050-101	
LS separation column	132-042-401	
Pre-separation filters	130-041-407	
Promega GoScript Reverse Transcription System	A5000	MyBio, Kilkenny, Ireland
Live H1N1(A/PR/8/34)	N/A	NIBSC, UK
Live H3N2(A/Uruguay/716/2007)	N/A	
RNeasy Plus Mini Kit	74134	Qiagen, Manchester, UK
Qiashredder	97654	
RNase-Free DNase Set	79254	R&D Systems, UK
Human IL-10 Duoset ELISA	DY217B	
Human IL-1β Duoset ELISA	DY201	
Human IL-27 Duoset ELISA	DY2526	
Human TGF-β Duoset ELISA	DY240	
Human IL-17A Duoset ELISA	DY317	
Human IFN-γ Duoset ELISA	DY285B	
Faststart Essential DNA Probes Master	06402682001	Roche Diagnostics, UK
Lightcycler 8-Tube Strips (Clear)	06327672001	
UPL Human GAPD Gene Assay	05190541001	
50 ml falcon tubes (non-skirted)	62.547.004	Sarstedt, Wexford, Ireland
50 ml falcon tubes (skirted)	62.559.001	
15 ml polypropylene tubes	62.554.001	
0.2 μm filters	83.1826.001	
1.5 ml screw-cap microtube	72.692.005	
5 ml pipette	86.1253.001	

10 ml pipette	86.1254.001	
25 ml pipette	86.1685.001	
Pipette tip, 10 μl	70.113.0	
Pipette tip, 20-200 μl	70.760.012	
Pipette tip, 100-1,000 μl	70.762	
T75 Suspension flask	83.3911.502	
Cardboard boxes	95.64.997	
24-well suspension tissue culture plate	83.3922.500	
24-well adherent tissue culture plate	83.3922	
0.2 mL Biosphere PCR tubes	72.737	
Filter tips 0.1-20 μl	70.1116.210	
Filter tips 2-200 μl	70.760.211	
Filter tips 100-1000 μl	70.762.211	
Ethelenediaminetetraacetic acid (EDTA)	AC09656	Scharlau, Chemie, S.A., Barcelona, Spain
Potassium chloride (KCl)	P00200	
Hepes	H4034	Sigma Aldrich, Wicklow, Ireland
Acridine Orange (Ao)	A9231	
Trizol	T9424	
TMB Substrate Solution	T8665	
Tween-20	P1379	
Sodium phosphate dibasic (Na_2HPO_4)	71636	
2-Mercaptoethanol	M3148	
2-Propanol for molecular biology, 99.5%	I9516-4X25ML	
Ethanol BioUltra for molecular biology, 99.8%	51976-500ML-F	
Universal Probe Library Probe 09	4685075001	
BD Cell Strainers	352340	Unitech, Dublin, Ireland

Table 2.2 List of equipment used in this study

Equipment	Model	Company
Flow Cytometer	FACSCalibur	BD Becton Dickson, UK
Steri-cycle CO_2 incubator	Hepa Class 100	Bio-sciences Ltd., Dun Laoghaire, Ireland
PowerEase® 90W Power Supply (230 VAC)	PS0091	
X25 Pipette Filter	10193923	Fisher Scientific, Dublin, Ireland
MJ Research Inc. Thermal Cycler	PTC-100	Lab Care Service, UK
Nanodrop Spectrophotometer	ND-1000	Mason Technology, Dublin, Ireland
Midi MACS Separation Unit	130-042-302	Miltenyi Biotec, Gladbach, Germany
MACS Multistand	130-042-303	
Haemocytometer	Improved, 2 grids	Neaubaurer
Fluorescent microscope	Eclipse E200	Nikon, U.S.A.
Alpina BIO1300 Class II Microbiological Safety Cabinet	50138	NSP Laboratory, Meath, Ireland
Lightcycler Nano qPCR Instrument	04717651001	Roche Diagnostics, UK
Four-digit hand tally counter	Z169021-1EA	Sigma Aldrich, Wicklow, Ireland
Centrifuge	Hettich Universal 320R	
Plate reader	Victor 2	Perkin Elmer
Heraeus centrifuge	Fresco 17	ThermoScientific

2.2.1 ISOLATION OF PRIMARY HUMAN IMMUNE CELLS

Buffy coats from healthy donors were obtained from the Irish Blood Transfusion service (at St James' hospital, Dublin). The peripheral venous blood (approximately 50 ml) was mixed with 5 ml of a 5% solution of EDTA in 1X PBS and diluted 1:2 with HBSS containing 1% FBS, and 10 μM HEPES buffer. This diluted blood was layered onto 14 ml of density gradient medium Lymphoprep™ (Axis-shield, Norway) and centrifuged at 400 x g for 25 minutes (with accelerator and break switched off to prevent the layers from mixing). Lymphoprep™ has a density of 1.077 g/ml and provides a gradient which enables the blood components to be separated according to their density. During centrifugation, erythrocytes and granulocytes sediment through the Lymphoprep™ to the bottom of the tube due to their higher density. The lower density mononuclear cells form a discrete cloudy layer at the interface of the sample. With blood components separated according to density, the buffy coat layer was removed using a Pasteur pipette and the cells were washed twice with 10 ml of supplemented HBSS, with centrifugation at 800 x g for 5 minutes. Cell pellets were resuspended in 5 ml of complete Roswell Park

Memorial Institute (cRPMI)-1640 medium (RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, and 100U penicillin/ml). To remove clumps from the peripheral blood mononuclear cells (PBMCs), the cells were filtered through a 40 µm filter and washed through with cRPMI. The cell yield and viability was calculated as per Section 2.2.2.

2.2.2 DETERMINATION OF CELL VIABILITY AND YIELD

2.2.2.1 Background

Acridine orange (AO) is a cell permeable nucleic acid binding dye. It emits green fluorescence under halogen or UV light when bound to double stranded DNA (dsDNA) of live cells, and emits a red fluorescence when bound to single stranded DNA or RNA. Thus, live cells will appear green, and non-viable cells will appear red/orange [360,361]. Ethidium bromide (EtBr) is a DNA intercalator, which inserts itself between the base pairs in the double helix. It stains dsDNA and under halogen or UV light, fluoresces orange. EtBr only stains cells with permeable membranes (such as cells in the final stages of apoptosis), and therefore can detect dead cells.

2.2.2.2 Method

An EtBr/AO solution was made by adding EtBr stock solution (0.8 ml of 4 mg/ml solution) to AO stock solution (2 ml of 1 mg/ml solution). The solution was made up to 200 ml with Sodium chloride (NaCl) with 0.85% weight/volume. The Improved Neubauer Haemocytometer slide was used to determine the number of cells in a specified volume. The haemocytometer slide contains a grid etched into its surface, which consists of nine 1 mm^2 squares. The defined volume of an area in the grid can be calculated from the area of the grid and the height between the grid and the cover slip (0.1 mm) (Figure 2.1). Cells to be counted were diluted in EtBr/AO and pipetted onto the haemocytometer beneath a cover slip, ensuring the cell solution covered the total surface of the grid. The number of live (green) and dead (orange) cells was determined by counting the cells in the four corners of the grid. The yield was calculated using the following formula:

$$(\text{Average cell number}) \times (\text{dilution factor}) \times 10^4 = \text{Cell number/ml}$$

The 10^4 value is the volume correction factor for the haemocytometer (each square is 1 mm^2 and the depth is 0.1 mm

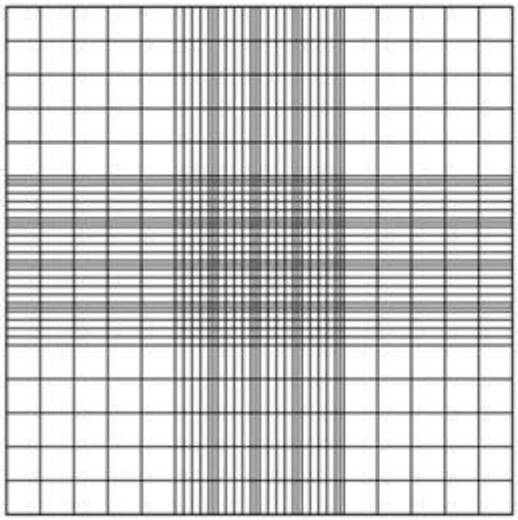

Figure 2.1 The grid of the Improved Neubauer Haemocytometer. The grid is etched onto the surface of the haemocytometer. The cells present in a certain area can be counted and based on the volume of the area, the cell number can be calculated. Image taken from Haemocytometer Counting Chambers [362].

2.2.3 SEPARATION OF CD14$^+$ AND CD3$^+$ CELLS FROM PBMCs USING MICROBEAD SEPARATION

2.2.3.1 Background

Magnetic microbead separation is based on antibodies coupled to magnetic beads. The conjugated antibodies are incubated with a cell suspension, and bind to the cells expressing the corresponding epitope. After incubation, the cell suspension is passed through a column which is placed in a magnetic field (MidiMAC). Magnetically labelled cells are retained in the column due to magnetic forces, and unlabelled cells flow through the column. To recover the labelled cells from the column, the column is removed from the magnetic field and the cells are forced through the column using a plunger and collected (Figure 2.2) [363].

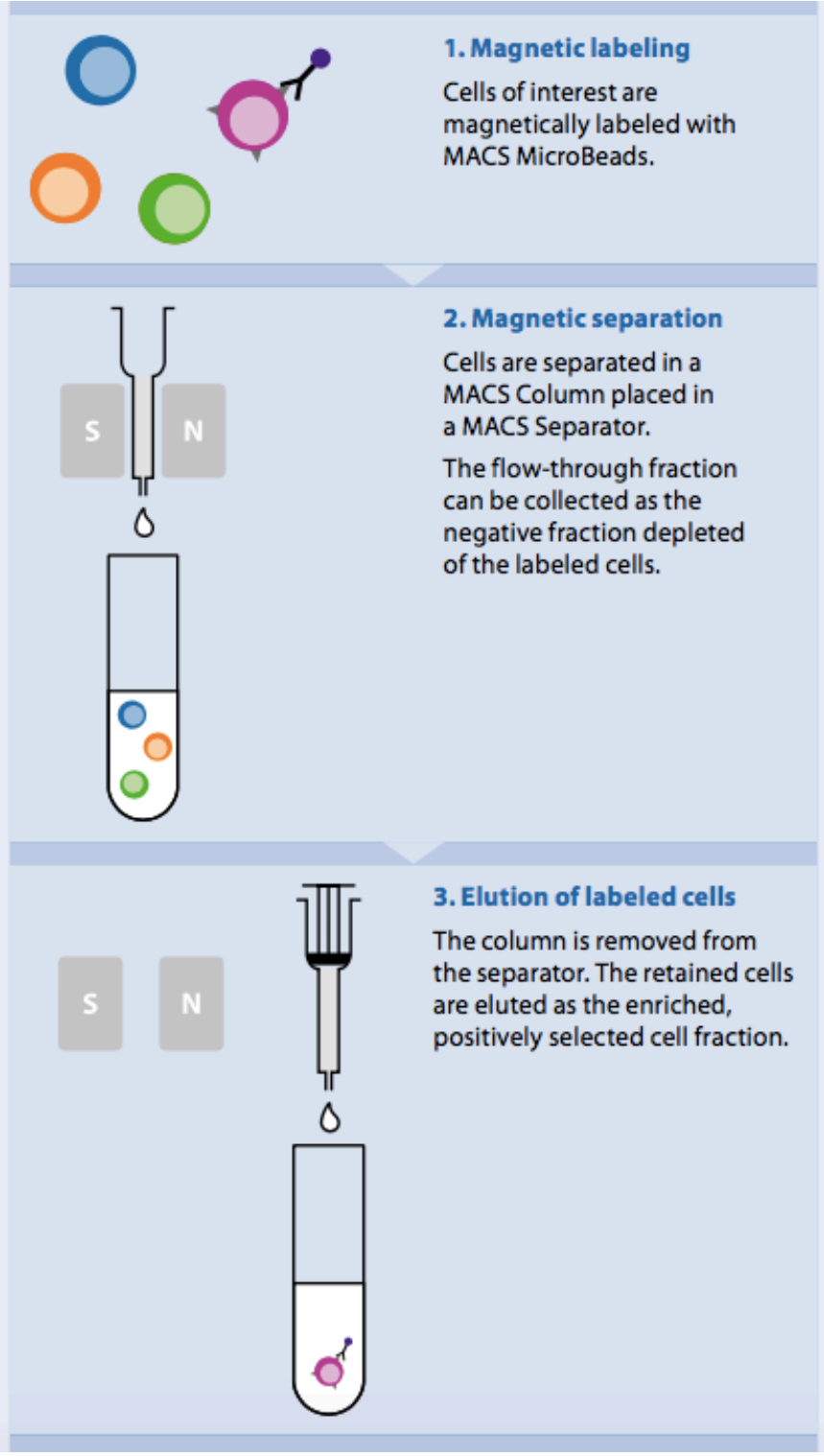

Figure 2.2 The principles of magnetic bead separation. 1. Cells of interest are labelled with MACS microbeads; 2. Cells are separated in a column; 3. Positively labelled cells are collected. Image taken from MACS Miltenyi [363].

2.2.3.2 Method

$CD14^+$ APCs and $CD3^+$ T cells were separated from human PBMCs, using $CD14^+$ microbeads and $CD3^+$ microbeads, respectively by MACS in accordance with manufacturer's instructions (Miltenyi Biotec, UK). Briefly, PBMCs were centrifuged at 800 x g for 5 minutes and resuspended in MACs buffer (sterile 1X PBS supplemented with 0.5% BSA and 2 mM EDTA) (80 μl of buffer/$1x10^7$ cells), and incubated with 150-250 μl of $CD14^+$ or $CD3^+$ microbeads for 30 minutes at 4°C. Following incubation, cells were washed with 5 ml of MACs buffer, and centrifuged at 800 x g for 5 minutes. The pellet was resuspended in MACs buffer (500 μl of buffer/$1x10^8$ cells). For positive selection of $CD14^+$ APCs or $CD3^+$ T cells, LS columns were attached to a magnet. The column was washed with 3 ml of MACs buffer, and the cell suspension was added to the column. The column was washed three times with 3 ml of MACs buffer. After all the MACs buffer had eluted through the column, the column was removed from the magnetic field, and the positively labelled cells were flushed out of the column with a plunger. Cells were centrifuged at 800 x g for 5 minutes and pellet was resuspended in appropriate volume of cRPMI to give a concentration of $1x10^6$ cells/ml.

2.2.4 VIRUS INFECTION OF $CD14^+$ ANTIGEN PRESENTING CELLS

$CD14^+$ APCs which had been separated from PBMCs (as per Section 2.2.3.2) were cultured in cRPMI at a concentration of $1x10^6$ cells/ml. Two strains of live IAV were used in this study: H1N1 (A/Puerto-Rico/8/34) and H3N2 (A/Uruguay/716/2007) provided by Ruth Harvey (NIBSC, UK). $CD14^+$ APCs at a density of $1x10^6$ cells/ml were infected with IAV for 2 hours at 37°C, then subsequently washed (centrifuged at 3,000 x g for 5 minutes) following incubation to remove excess virus. The cells were resuspended in 1 ml of fresh cRPMI and cultured on a 24-well plate for 24 hours, alone, or in combination with Heat Killed *S.p*. (as per Section 2.2.5).

2.2.5 STREPTOCOCCUS PNEUMONIAE STIMULATION OF CD14$^+$ APCs

CD14$^+$ APCs at a density of 1x10^6 cells/ml which had been separated from PBMCs (as per Section 2.2.3.2) were exposed to Heat Killed *S.p.* (HKSP) (10^7 CFU) (Invivogen), alone or in combination with live IAV-infected APCs (as per Section 2.2.4) for 24 hours.

2.2.6 CO-CULTURE ASSAY

2.2.6.1 Background

Mixed lymphocyte reaction (MLR) is an assay commonly used to examine T cell responses. In a MLR, CD3$^+$ T cells isolated from one donor are co-cultured with CD14$^+$ APCs from a different donor (Figure 2.3). During co-culture, the CD3$^+$ T cells will proliferate in response to the "non-self" MHC molecules from the CD14$^+$ APCs [116].

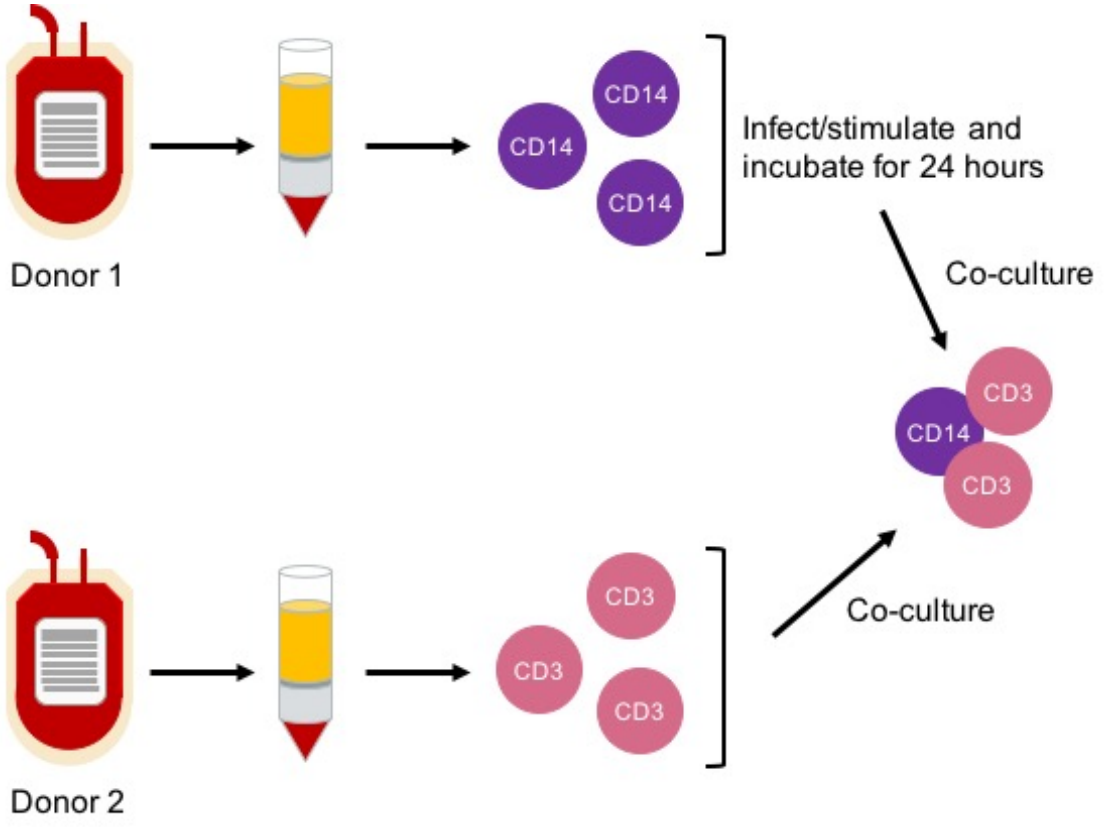

Figure 2.3 Scheme of Mixed Lymphocyte Reaction. A blood donation obtained from a healthy human donor is used to isolate PBMCs using gradient centrifugation. CD14$^+$ APCs are separated from PBMCs, infected/stimulated and incubated for 24 hours. A blood donation from a different healthy human donor is used to isolate PBMCs. CD3$^+$ T cells are separated from PBMCs. CD14$^+$ APCs are removed from incubation, and co-cultured with CD3$^+$ T cells for a further 24 hours.

2.2.6.2 Method

After incubating $CD14^+$ APC for 20-24 hours as described before, cells were centrifuged at 2,000 x g for 5 minutes. These cells were resuspended in cRPMI and seeded in 24 well cell culture adherent plates. Co-cultures of $CD3^+$ T cells and $CD14^+$ APCs were performed at 5:1 ratios ($1x10^6$ cells/ml of $CD3^+$ T cells and $2x10^5$ cells/ml of $CD14^+$ APCs). As a negative control, $CD3^+$ T cells alone were also cultured. Co-cultures were incubated for 24 hours at 37°C. All experiments were performed in triplicate on adherent 24 well cell culture plates.

2.2.7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.2.7.1 Background

Sandwich ELISA measures the amount of cytokine produced in the cells supernatant. A fixed quantity of capture antibody (mAb), specific for the cytokine being detected is bound to a 96-well plate. The capture antibody is diluted in buffer and incubated overnight. The plate is washed to remove unbound antibody and a blocking buffer is added to prevent non-specific binding. The plate is washed again and recombinant cytokine standards of known concentration, and samples of unknown concentration are added to the plate. The standards and samples can either be incubated overnight at 4°C or incubated for 2 hours at room temperature. If present, the cytokine of interest binds to the capture antibody during incubation. The plate is washed to remove unbound antigens or cytokines and a biotinylated detection antibody specific for the cytokine is added to the plate and incubated for 2 hours at room temperature. The detection antibody binds to the cytokine of interest during incubation. The plate is washed again and streptavidin-horseradish-peroxidase (HRP) is added to the plate. During incubation, streptavidin binds to biotin with high affinity and is conjugated to HRP. Following HRP incubation, the plate is washed a final time and the substrate, tetramethylbenzidine (TMB), is added to the plate. As HRP is an enzyme which catalyses the oxidation of its substrate TMB by hydrogen peroxide, a blue compound will be formed if the cytokine-antibody is present. The intensity of the blue colour increases with the concentration of the cytokine of interest. See Figure 2.4 for a workflow of the ELISA methodology. The reaction is stopped by the addition of a stop solution (sulphuric acid) and the absorbance/optical density of the samples is measured at 450 nm using a plate reader. The absorbance is proportional to the concentration of the cytokine in the sample. The cytokine standard concentrations are plotted against the absorbance to produce a standard curve, from

which the concentrations of the unknown samples can be extrapolated from the curve using their absorbance readings.

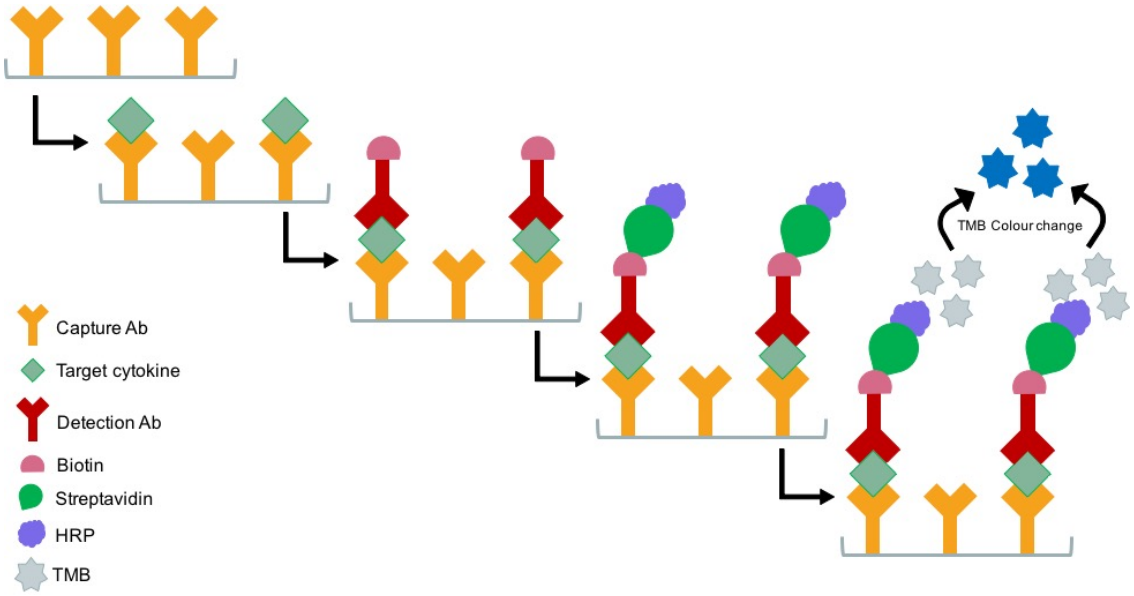

Figure 2.4 Workflow of a sandwich ELISA principle. Capture Antibodies are coated in the well and any antigen/cytokine present in a sample binds to the capture antibody. A biotinylated detection antibody binds to the immobilised antigen/cytokine and an enzyme binds to the mobilised detection antibody. A substrate is added which forms a coloured product. The rate of colour changes is proportional to the concentration of antigen/cytokine in the sample.

2.2.7.2 Method

Supernatant from treated cells (as above) was used to detect for the following cytokines using ELISA kits; IL-23, IL-12p70 (Biosciences), IL-6, IL-1β, IL-27, TGF-β, IL-10, IL-17A, and IFN-γ (R&D systems) according to manufacturer's protocol. Samples and standards were plated either in duplicate to ensure accurate quantitative results were obtained.

2.2.8 REAL TIME POLYMERASE CHAIN REACTION (qPCR)

2.2.8.1 Background

PCR is a method for synthesising and amplifying specific DNA sequences. To begin, the DNA is denatured by heating to 95°C, which separates the DNA into single strands of DNA. Primers for the PCR reaction are designed against a stretch of nucleotides within the target gene sequence. These primers anneal to their complementary DNA strands when the mixture is heated to the appropriate temperature (usually 50-60°C) and flank the target DNA sequence to be amplified. A DNA polymerase enzyme such as *Taq*

polymerase catalyses the elongation of the primers when the mixture is heated. As the enzyme is thermostable, it can withstand the high temperatures required during the PCR. The denaturation, annealing, and elongation steps are cycled numerous times in order to amplify the PCR product.

Real Time PCR (qPCR) provides relative and absolute quantification of gene expression. Small amounts of cDNA are amplified using the same methodology as conventional PCR.

In qPCR, a fluorophore and primers, which will bind to the amplified PCR product are added to the mixture, in the thermal cycler machine that contains sensors which can measure fluorescence (Figure 2.5). The fluorophore will fluoresce after it has been excited at the appropriate wavelength, which can be measured, allowing the amplification of one or more products to be analysed at the same time. This data can subsequently be analysed to calculate the relative gene expression in multiple samples.

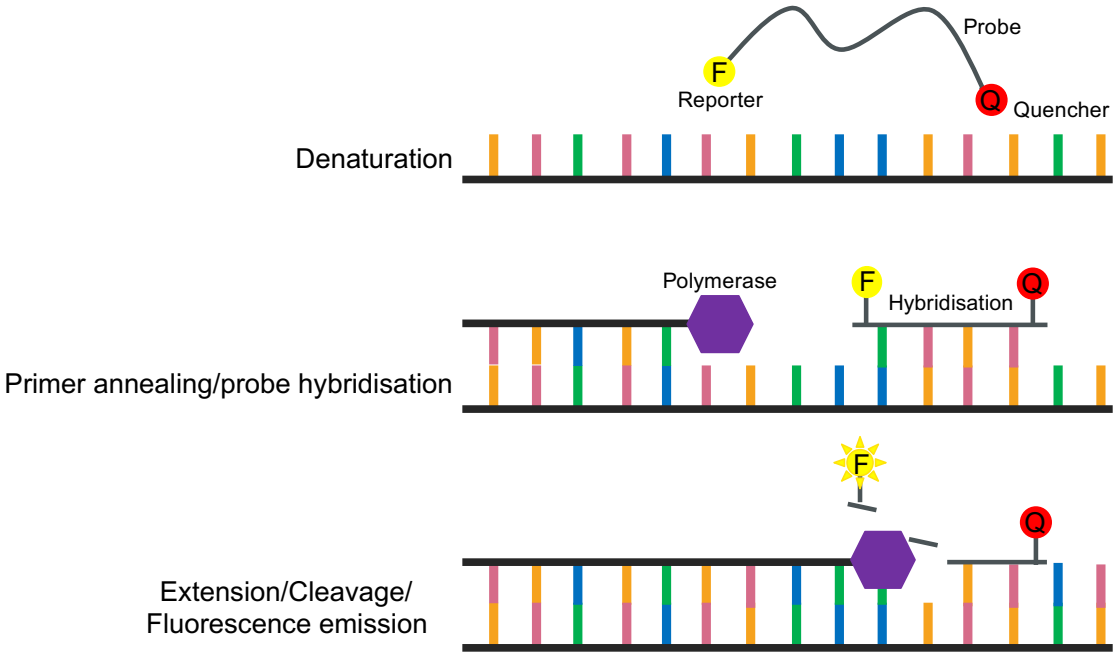

Figure 2.5 Schematic of probe-based qPCR. After DNA denaturation, the probes hybridise to the single-stranded DNA and the primers also anneal to the single-stranded DNA. As the new strand of DNA is extended, the probe is cleaved from the DNA, resulting in fluorescence emission.

2.2.8.2 RNA Extraction

To perform experiments such as quantitative polymerase chain reaction (qPCR), successful extraction of high quality RNA is critical. All work surfaces were covered with bench protector and cleaned using RNase Zap (Biosciences Ltd.) and 70% ethanol (Sigma Aldrich). RNase free filter pipette tips (Sarstedt) were used throughout. Appropriate PPE was worn and gloves were changed regularly. RNA extractions were

performed using either the RNeasy mini kit (Qiagen), including DNase digestion using the RNase-free DNase kit (Qiagen), or using the RNeasy Plus mini kit (Qiagen) as per manufacturer's instructions. Cells were lysed and homogenised using a QIAshredder (Qiagen) and a vortex. Ethanol is added to the lysate which provides ideal binding conditions. The lysate is loaded into the RNeasy silica membrane and the RNA binds to it. The RNeasy Plus mini kit (Qiagen) removes genomic DNA contamination using a gDNA Eliminator spin column, removing the need for separate DNase treatments (Qiagen, 2017). RNA was eluted in 30 µl of nuclease-free water (Biosciences Ltd.).

2.2.8.3 RNA Quantification and Determination of Purity

Extracted RNA (1 µl) was quantified using a Nanodrop Spectrophotometer (Mason Technology) at 260 nm and 280 nm. Purity of RNA was estimated using the A_{260}:A_{280} ratio and the A_{260}:A_{230} ratio. Pure RNA will have a A_{260}:A_{280} ratio of ~2.0 and a A_{260}:A_{230} ratio of 1.8-2.2 [364]. Isolated RNA was stored at -80°C.

2.2.8.4 cDNA Synthesis

2.2.8.4.1 Background

cDNA synthesis is performed using the enzyme reverse transcriptase, which creates complementary DNA based on the pairing of RNA base pairs to the DNA complements. Total RNA (extracted as per Section 2.2.8) was used in cDNA synthesis performed using the GoScript Reverse Transcription System (Promega) as per manufacturer's instructions, with deviations as below.

2.2.8.4.2 Method

Mastermix was prepared as per Table 2.3 and Table 2.4.

Table 2.3 Components and volumes used in preparation of RNA and primer mix

Component	**Volume (µl)**
Experimental RNA*	X
Oligo(dT) primer	1.0
Random primer	1.0
Nuclease-Free Water*	X
Total Volume	11.5

*Volume depended on specific RNA concentration of each sample. At least 100 ng of RNA was used per reaction

RNA/primer mix was heated at 70°C for 5 minutes and immediately chilled in ice water for another 5 minutes. Mixture was centrifuged for 10 seconds and stored on ice until reverse transcription mix was added.

Table 2.4 Components and volumes used in preparation of cDNA reverse transcription mix

Component	Volume (µl)
GoScript™ 5X Reaction Buffer	4
$MgCl_2$	1.5
PCR Nucleotide Mix	1.0
Recombinant RNasin® Ribonuclease Inhibitor	1.0
GoScript™ Reverse Transcriptase	1.0
Total Volume	8.5

RNA and primer mix (11.5 µl) was combined with reverse transcription mix (8.5 µl). The mixture was heated to allow for annealing in a heat block at 25°C for 5 minutes; heated to allow for extension in a heat block at 42°C for 1 hour; and the reverse transcriptase was inactivated in a heat block at 70°C for 15 minutes. Samples were frozen at -20°C.

2.2.8.5 qPCR Method

cDNA samples were amplified using the Lightcycler Nano (Roche Diagnostics) with the Faststart Essential DNA Probes Master System (Roche Diagnostics) and RealTime Ready Assays (Roche Diagostics/Sigma Aldrich). Expression of H1N1 nucleoprotein (NP) and H3N2 NP using a probe (Sigma-Aldrich) and primer sequences as below (Table 2.5), was normalised to the expression of the reference gene, GAPDH.

Table 2.5 Primer Sequences for H1N1 and H3N2 Nucleoprotein

H1N1 NP Forward Primer	GGTGCTGCAGTCAAAGGAGT
H1N1 NP Reverse Primer	CCCACGTTTGATCATTCTGA
H3N2 NP Forward Primer	GGTGCTGCAGTCAAAGGAAT
H3N2 NP Reverse Primer	CCCCGTTTGACCATTCTG

A 20 μl volume reaction was set up for each sample using 2 μl of cDNA as a template. The reaction was set up as follows:

Table 2.6 Reagents and volumes used in preparation of qPCR experiment

Reagent	Volume (μl)
Probes Master Mix	10.0
Gene of Interest Forward Primer	0.5
Gene of Interest Reverse Primer	0.5
Gene of Interest Probe	1.0
Reference Gene Primer	0.5
Reference Gene Probe	0.5
cDNA	X
PCR Grade Water	X

The following run settings were set up on the Lightcycler Nano:

1. Hold at 95°C for 600 seconds (ramp 4°C/s)
2. Two-step amplification repeated for 50 cycles:
    a. 95°C for 10/20 seconds (ramp 5°C/s)
    b. 60°C for 30/40 seconds (ramp 4°C/s)
3. Hold at 40°C for 30 seconds (ramp 2.2°C/s)

2.2.9 APOPTOSIS STUDY USING FLOW CYTOMETRY

2.2.9.1 Background

Flow cytometry is a technique used to rapidly analyse various characteristics of individual cells as they pass laser beams of light. This technique produces both quantitative and qualitative data, including information about cell size, DNA/RNA content, and expression of protein markers. To analyse protein expression, cells are incubated with antibodies conjugated to fluorescent dyes which bind to the protein of interest. When these labelled cells pass by a light source, the fluorochromes are excited to a higher energy state resulting in an emission of light energy, enabling detection of protein. Multiple fluorochromes can be used to measure several cell properties at once. During analysis, cell suspension which have been labelled with fluorochomes are forced into a laminar flow stream which is created by sheath fluid. The laminar flow enables the cells to be individually spaced in the stream of liquid. As each cell passes the laser beam, the cell scatters the laser light and the fluorochromes fluoresce at different wavelengths. The scattered light is detected by photomultiplier tubes and subsequently digitised (Figure 2.6) [365]. Analysis of apoptosis can be achieved using flow cytometry by using stains

such as Annexin V and propidium iodide (PI). In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated to the outer plasma membrane and exposed to the external cellular environment. Annexin V is a phospholipid-binding protein which has a high affinity for PS, and binds to cells with exposed PS. Annexin V can be used as a probe in flow cytometry when conjugated to fluorescein isothiocyanate (FITC), a commonly used fluorochrome. FITC Annexin V staining can identify early apoptosis as externalisation of PS occurs in the early stages of apoptosis. The vital dye, PI, can be used to identify late apoptosis or necrosis. Viable cells which have their membranes intact exclude PI are not permeable to PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, viable cells will be FITC Annexin V and PI negative, cells in early apoptosis are FITC Annexin V positive but PI negative, and cells in late apoptosis/necrosis are both FITC Annexin V and PI positive [366–369].

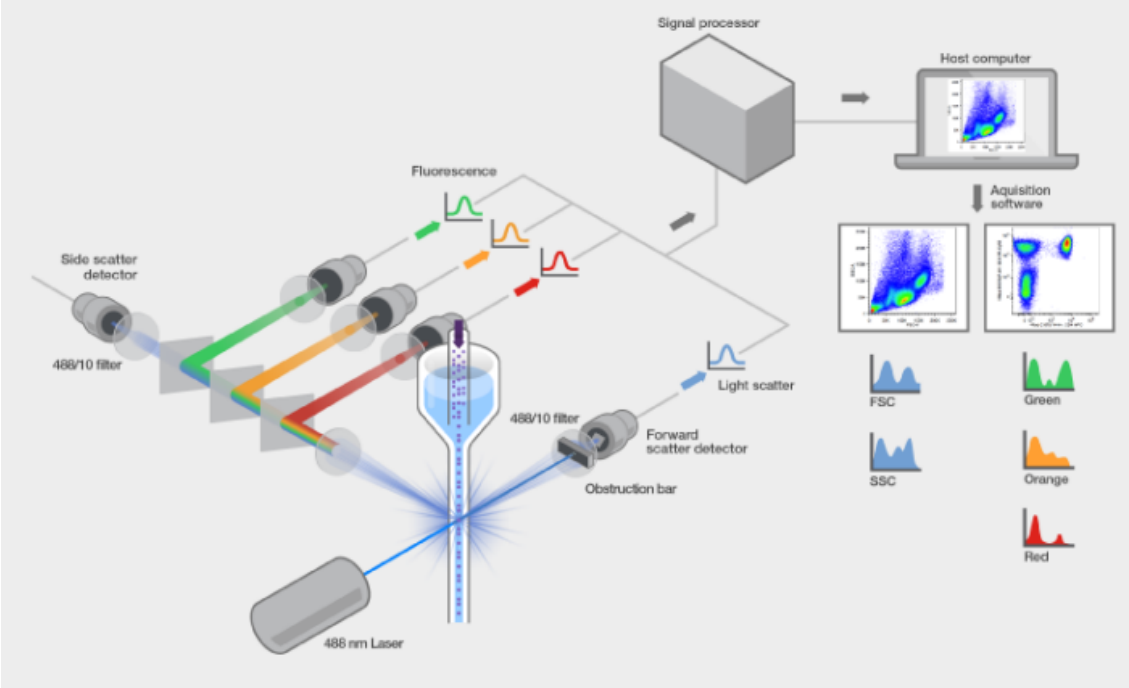

Figure 2.6 Schematic displaying the principles of flow cytometry. The sample is transported from the sample tube through the flow cytometer for analysis through sheath fluid. At the interrogation point, the cell interacts with the laser light which causes the light to scatter. The light scatter can be measured and are called forward angle scatter (FSC) and side angle scatter (SSC). The laser light will also excite any fluorophores associated with the cell, resulting in fluorescence emission, which is collected by the detector and processed using software. Image taken from ThermoFisher [370].

2.2.9.2 Method

Apoptosis studies were performed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) as per manufacturer's instructions. $CD14^+$ cells were centrifuged at

2,000 x g for 5 minutes and washed twice in ice-cold 1X PBS. Cells were resuspended in 1X Binding buffer at a concentration of $1x10^6$ cells/ml and 100 μl of the solution ($1x10^5$ cells) was transferred to a 5 ml culture tube and 5 μl of both FITC Annexin V and PI were added to the solution. The solution was vortexed and incubated at RT in the dark for 25 minutes. 1X Binding buffer (400 μl) was added to each tube and the solutions were analysed using the flow cytometer, FACSCalibur (BD Becton Dickenson). Data was analysed using Cellquest software. The following controls were used to set up compensation and quadrants: unstained cells, cells stained with FITC Annexin V (no PI), cells stained with PI (no FITC Annexin V).

2.2.10 WESTERN BLOT

2.2.10.1 Background

Western blot is a common method used to detect specific proteins in a cell lysate. Initially, cell lysates are prepared from cells using extraction buffer and the protein concentration is quantified. The protein extract is diluted with loading buffer, which consists of glycerol (which aids the loading of the sample into the gel), and a dye (such as bromophenol blue, which is added to visualise the sample). The sample is heated to fully denature the proteins and subsequently loaded to separate the proteins according to size by SDS-PAGE. After gel electrophoresis, the proteins are transferred to a stable support membrane (such as nitrocellulose). During the transfer, voltage is applied to transfer the proteins from the gel to the membrane. Once the proteins are transferred, the membrane is incubated in a blocking buffer (made with BSA or non-fat dried milk) to prevent non-specific binding. Specific proteins are then detected by primary antibodies which bind to their corresponding protein on the membrane. The primary antibody is then detected by a secondary antibody, which is usually a horseradish-peroxidase-linked secondary antibody. When a chemiluminescent agent is added to this, a reaction occurs, forming a luminescent product proportional to the amount of protein. This can be imaged using a variety of techniques including using photographic film which when placed on the membrane is exposed to the light from the reaction, creating an image of the antibodies bound to the blot (Figure 2.7) [116,371,372].

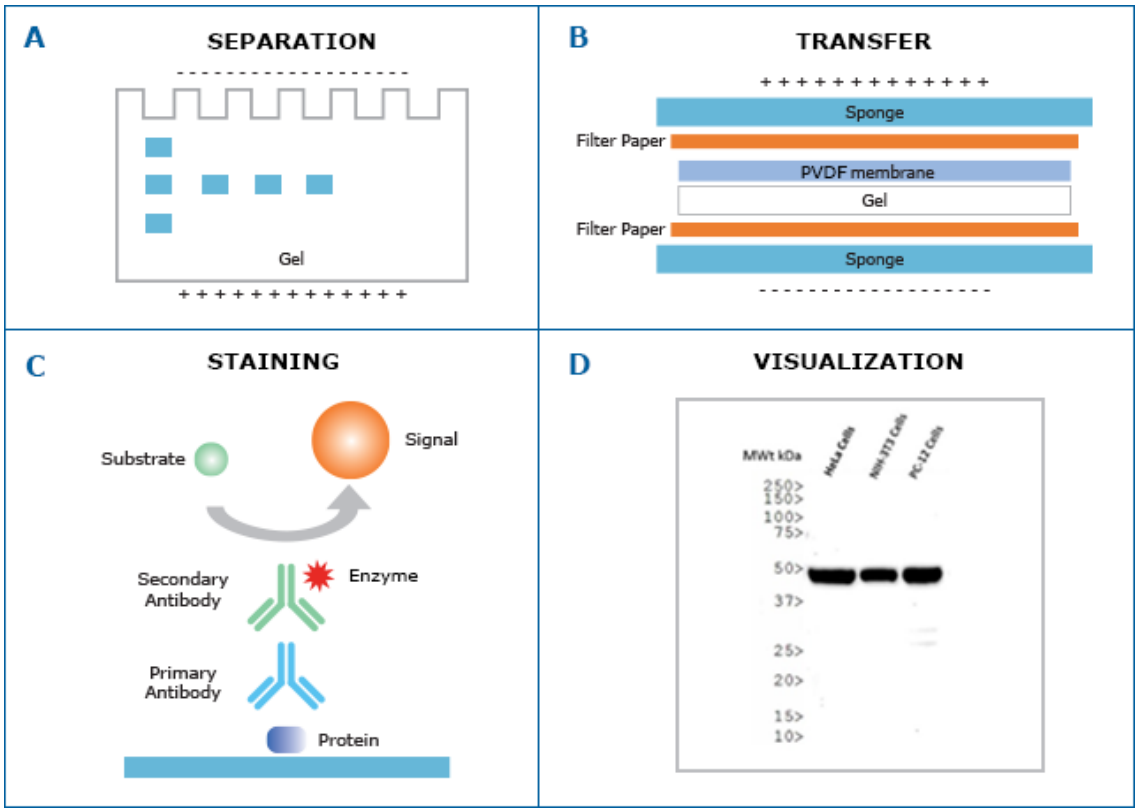

Figure 2.7 The Stages of Western Blot. (A) Separation of protein by SDS-PAGE. (B) Proteins are transferred from the gel to the membrane. (C) The protein on the membrane is detected using primary and secondary antibodies, which reacts with an enzyme to emit light. (D) The proteins are imaged. Image taken from Novus Biologics [373].

2.2.10.2 Method

Cells were lysed on ice in radio-immunoprecipitation assay (RIPA) lysis and extraction buffer supplemented with 1X Halt protease and phophatase inhibitor cocktail (ThermoFisher Scientific). Cell suspension was vortexed and spun in a centrifuge for 13,300 x g for 15 minutes at 4°C. Supernatant was removed and frozen at -80°C. Protein samples were quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) as per manufacturer's instructions. 4X Bolt LDS Sample Buffer and 10X Bolt Reducing Agent was added to protein samples with deionised water to make final volume up to 40 μl. Samples were boiled at 95°C for 5 minutes. Samples were loaded and SDS-PAGE was performed using Bolt 4-12% Bis-Tris Plus Gels. Proteins were transferred to 0.45 μm PVDF membrane and blocked in 5% non-fat dry milk (NFDM) in TBS-T for 2 hours at room temperature and probed with Human Anti-β-Actin (Abcam) (1/5000 dilution) in 5% NFDM in TBS-T overnight at 4°C. Membrane was washed and incubated with Goat Anti-Rabbit IgG H&L (HRP) (Abcam) (1/10000 dilution) in TBS-T for 1 hour at

room temperature. Membrane was washed and incubated in SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific) for 5 minutes at room temperature and developed in a dark room.

2.2.11 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 6.0 for Mac (GraphPad Software). Data was normalised by setting untreated sample readings to ~1 and comparing treated sample readings to that value, thus providing relative concentrations. A One-Way ANOVA was fitted to the data and comparisons of interest were made using a Sidak test to adjust for multiple testing, using a 5% significance interval; p-values less than 0.05 were considered significant and are represented as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.3 RESULTS

2.3.1 CONFIRMATION AND QUANTIFICATION OF INFLUENZA A VIRUS INFECTION OF HUMAN CD14$^+$ APCs

The influenza nucleoprotein (NP) is a highly conserved viral protein, which is essential for viral replication. As NP is expressed intracellularly during influenza infection, it can serve as an indicator to confirm IAV infection [340,352]. To confirm that live IAV could infect CD14$^+$ APCs, RNA was isolated from virally infected CD14$^+$ APC cell pellets. cDNA synthesis was performed on isolated RNA and IAV NP expression was determined using qPCR as per Section 2.2.8. Primers specific for both H1N1 and H3N2 NP were chosen and amplification of the gene was normalised to gene expression of the housekeeping gene, GAPDH. No NP (H1N1 or H3N2) was detected in untreated cells. H1N1 NP was detected in H1N1-infected cells and H3N2 NP was detected in H3N2-infected cells (Figure 2.8). Expression of NP was very consistent between H1N1-infected donors and H3N2-infected donors, although higher levels of NP expression was detected in H1N1-infected donors.

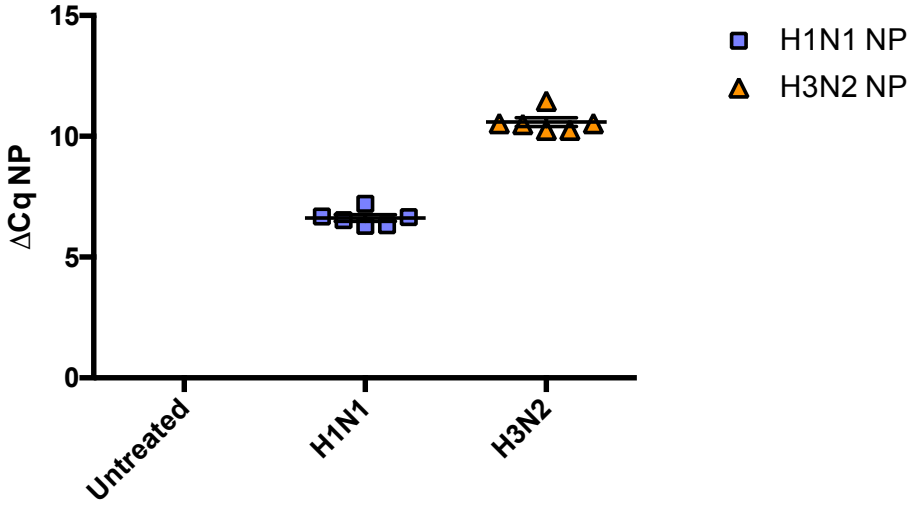

Figure 2.8 H1N1 and H3N2 are confirmed to infect CD14$^+$ APCs. The levels of H1N1 and H3N2 NP mRNA expression by CD14$^+$ cells following 24 hr treatment with live H1N1 or H3N2 or untreated as a control were determined by qPCR. Cq values of H1N1 and H3N2 NP were normalised to the expression of GAPDH and plotted on a scatter dot plot, where higher Cq values indicates lower abundance. Each range of dots represents normalised expression of NP + SEM of 2 experimental repeats of each treatment in the same donor (n=3). Fold expression was not calculated as NP was not amplified in untreated samples.

2.3.2 NEW BATCHES OF STREPTOCOCCUS PNEUMONIAE INDUCE INNATE CYTOKINES

Previously *S.p.* was cultured and heat-killed by personnel in the lab [350]. However, for these studies, commercially bought HKSP (Invivogen) was used throughout. The level of induction of new batches was compared to previous results by cross-checking fold-change in cytokine expression of HKSP-treated cells relative to untreated cells, which were normalised to ~1.0 (Table 2.7). The level of HKSP induction of IL-23, IL-6, and TGF-β were very similar between batches of HKSP. The level of induction of IL-27, IL-12p70, IL-10, and IL-1β by the new batch of HKSP was not as high as the previous one, however strong induction still occurred.

Table 2.7 Comparison of levels of induction between batches of Heat Killed *Streptococcus pneumoniae*

Cytokine	Previous HKSP*	New HKSP**
IL-23	2.27	2.64
IL-6	1.13	1.04
IL-27	2.18	1.35
IL-12p70	4.36	1.32
IL-1β	3.00	1.84
IL-10	3.57	1.58
TGF-β	0.92	0.96

*n=9; **20

2.3.3 A NEW BATCH AND CLINICAL ISOLATE OF INFLUENZA A VIRUS INHIBIT INNATE TH17 AND TH1 POLARISING CYTOKINE RESPONSES TO STREPTOCOCCUS PNEUMONIAE

It is widely accepted that IAV infection inhibits immune responses to *S.p.* in humans and mouse models [1–3,18]. Having confirmed the ability of IAV to infect isolated $CD14^+$ APCs (Figure 2.8), the effect of IAV on immune responses to *S.p.* was examined. Here, it is shown that IAV inhibits innate immune responses to *S.p.* in primary human immune cells. This inhibition has involved cytokines in the Th17 response. It should be noted that although IL-27 is not traditionally known to be a Th17 cytokine, it has been shown that under certain circumstances, it is capable of inducing Th17 differentiation [182]. Supernatants from treated $CD14^+$ APCs were analysed for the following cytokines using ELISA (as per Section 2.2.7). Significant inhibition of HKSP-induced IL-23, IL-6, IL-27 (n=20) (Figure 2.9 A-C) by a new batch of H1N1 and a new clinical isolate of H3N2

occurred. Significant inhibition of HKSP-induced IL-12p70 (n=10) (Figure 2.9 D) by a new batch of H1N1. No inhibition of HKSP induced IL-1β by IAV occurred (Figure 2.10) (n=20).

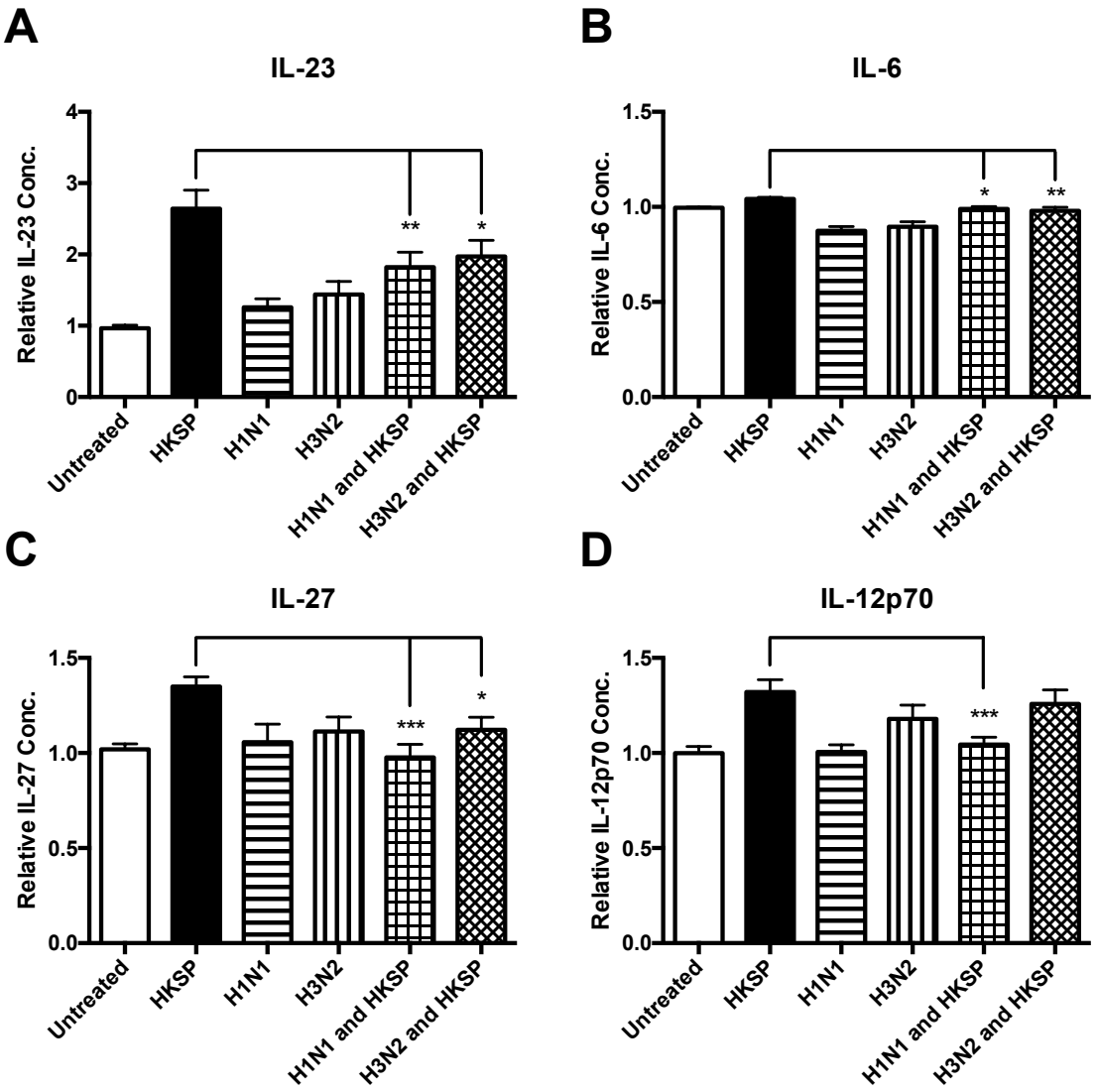

Figure 2.9 Live IAV infection inhibits HKSP-induced IL-23, IL-6, IL-27, and IL-12p70. The levels of (A) IL-23, (B) IL-6, (C) IL-27, and (D) IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 20 donors (n=20). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

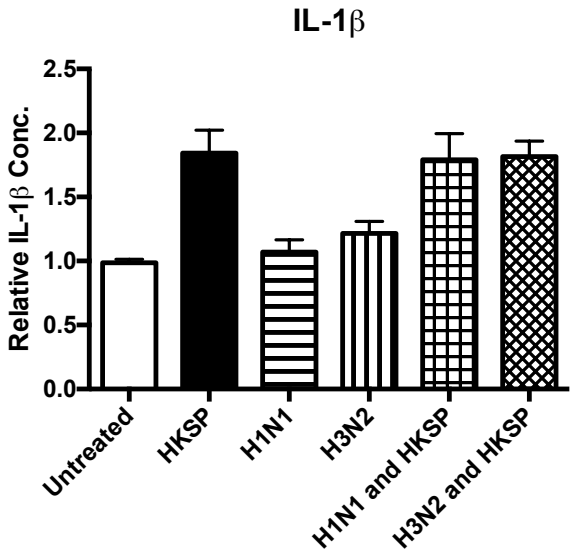

Figure 2.10 Live IAV infection does not inhibit HKSP-induced IL-1β. The levels of IL-1β secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 20 donors (n=20). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

2.3.4 INFLUENZA A VIRUS INHIBITION IS NOT DUE TO ANTI-INFLAMMATORY EFFECTS OF IL-10 AND TGF-β

In addition to a direct effect on Th17 related cytokines, the balance of anti-inflammatory (T regulatory responses) to pro-inflammatory responses can impact on the development of protective Th17 responses to *S.p.* infection [89,208]. To assess if IAV altered the levels of HKSP-induced anti-inflammatory cytokines, we assessed the levels of IL-10 and TGF-β. IL-10 is an essential immune system regulator by inducing T_{reg} cells which limit inflammatory responses that could otherwise cause tissue damage [211,243,244]. TGF-β is a pleiotropic cytokine which acts as an immune modulator. In the presence of IL-6, TGF-β drives differentiation of Th17 cells, however it can also exert anti-inflammatory effects to prevent pathogenic effects of Th17 cells, which would inhibit the Th17 response [176,208].

Supernatants from treated $CD14^+$ APCs were used to detect for cytokines using ELISA (as per Section 2.2.7). H1N1 infection significantly inhibited HKSP-induced IL-10 (Figure 2.11 A) (n=20), although there was little difference between influenza strains. IAV did not inhibit HKSP-induced TGF-β (Figure 2.11 B) (n=20). The inhibition of the Th17 response

cannot be attributed to the anti-inflammatory effects of IL-10 and TGF-β as *S.p.* induction of these cytokines was not elevated. This further supports the previous conclusions that the inhibition of Th17 polarising responses is not due to an indirect effect of anti-inflammatory regulation.

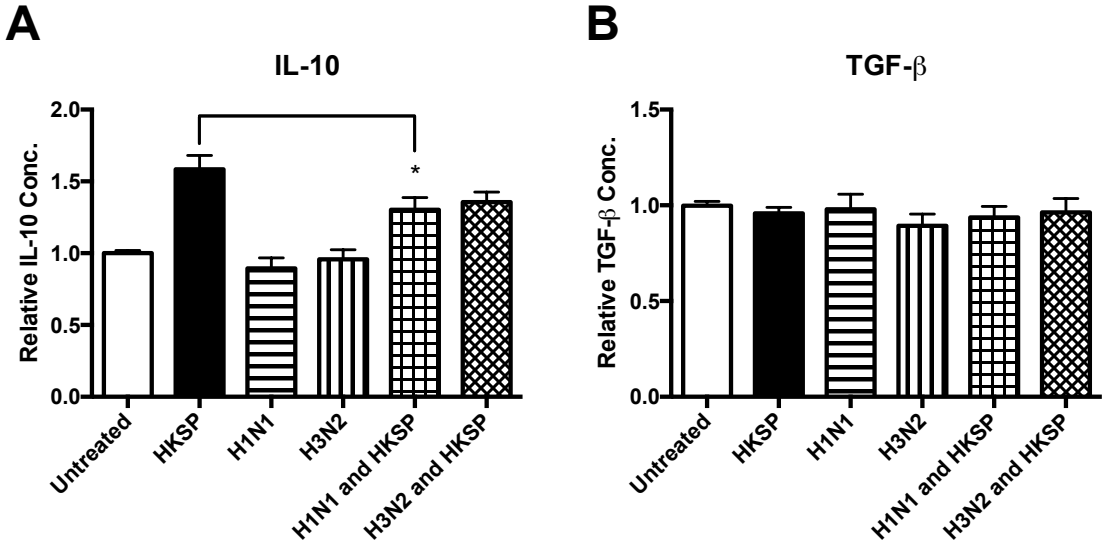

Figure 2.11 Live IAV infection does not increase anti-inflammatory cytokines. The levels of (A) IL-10 and (B) TGF-β secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 20 donors (n=20). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*$p<0.05$).

2.3.5 A NEW BATCH AND CLINICAL ISOLATE OF INFLUENZA A VIRUS HAVE SIMILAR IMMUNOSUPPRESSIVE EFFECTS TO PREVIOUS ISOLATES

Previous studies carried out in the Viral Immunology Lab demonstrated that IAV inhibited important pneumococcus driven cytokines in human APCs [350]. Upon receipt of a new batch of H1N1 and a new clinical isolate of H3N2 from the NIBSC, previous studies were repeated to further the human *ex vivo* immune model to determine if any differences occurred between IAV batches and clinical isolates (Table 2.8). The level of inhibition of HKSP responses by IAV was compared between different batches and clinical isolates by cross-checking differences in fold-change cytokine expression between HKSP-treated cells and HKSP and IAV co-treated cells. The new results obtained for IL-23, IL-

27, IL-1β, and TGF-β were in keeping with what was previously obtained. However, previously only H3N2 significantly inhibited HKSP-induced IL-6, whereas both new strains of IAV significantly inhibited HSKP-induced IL-6. Previously, both strains of IAV inhibited pneumococcus induction of IL-12p70, however only the new strain of H1N1 inhibited pneumococcus-driven IL-12p70. Additionally, the new strain of H1N1 also inhibited HKSP-induced IL-10, which was not previously shown.

Table 2.8 Comparison between inhibition of previous results against current results generated with new batches of H1N1 and clinical isolates of H3N2

Cytokine	H1N1		H3N2	
	Previous*	New**	Previous*	New**
IL-23	0.45	0.82	0.4	0.67
IL-6	0.11	0.05	0.12	0.06
IL-27	0.47	0.37	0.75	0.23
IL-1β	-0.02	0.05	0.19	0.03
IL-12p70	2.55	0.28	3.32	0.06
IL-10	-0.05	0.28	0.25	0.22
TGF-β	0.07	0.02	0.02	0.00

Yellow indicates inhibition of HKSP by IAV, whereas blue indicates no inhibition. Numerical values represent the difference between HKSP relative concentrations and HKSP and IAV relative concentrations. Negative numbers indicate that co-treated cells induced higher amounts of cytokine than HKSP alone treated cells. *n=9; **n=20

2.3.6 INHIBITION OF TH17 AND TH1 INNATE CYTOKINES IS NOT DUE TO APOPTOSIS OR PROTEIN SYNTHESIS SHUTDOWN

Apoptosis a method of "programmed" cell death and is a normal process which occurs during cell development, aging, and as a mechanism to maintain homeostasis. It can also occur as a defence mechanism in certain immune responses [374,375]. To ensure that the inhibition of Th17 and Th1 innate cytokines in our human *ex vivo* model was not due to apoptosis, an apoptosis study was performed as per Section 2.2.9. Levels of apoptosis were not increased in cells treated with HKSP and IAV, compared with HKSP alone. The Th17/Th1 response inhibition was not due to an increase in apoptosis (Figure 2.12). To ensure that inhibition of innate immune responses was not due to protein synthesis shutdown, a western blot was performed as per Section 2.2.10. As the housekeeping protein, β-Actin was detected in all cells, regardless of treatment, this indicates that protein synthesis shutdown did not occur (Figure 2.13). This points to IAV infection having a direct effect on immune pathways which are key in clearing *S.p.* infection, which is not as a result of apoptosis or protein synthesis shutdown.

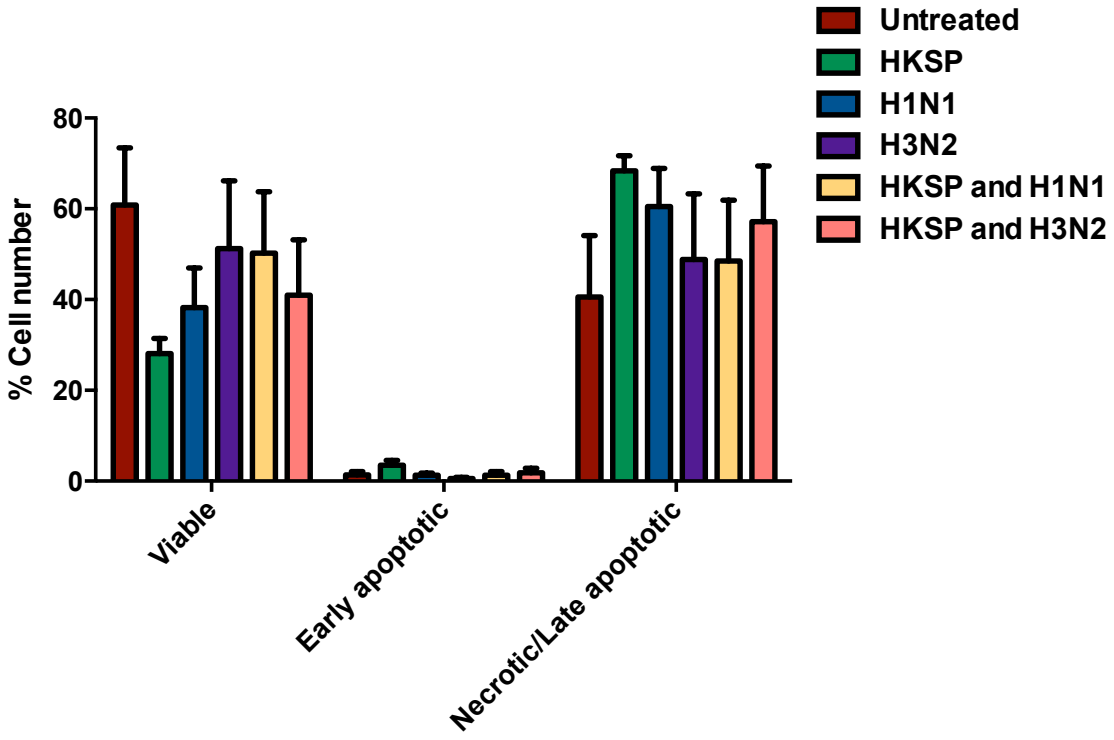

Figure 2.12 Inhibition by IAV of innate responses to *S.p.* is not due to apoptosis. $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP (or untreated as a control) were dual stained with FITC Annexin V and propidium iodide. The percentages of viable, early apoptotic, and necrotic/late apoptotic after treatments were determined using flow cytometry. Each column represents mean % cell number + SEM of 3 experimental donors (n=3).

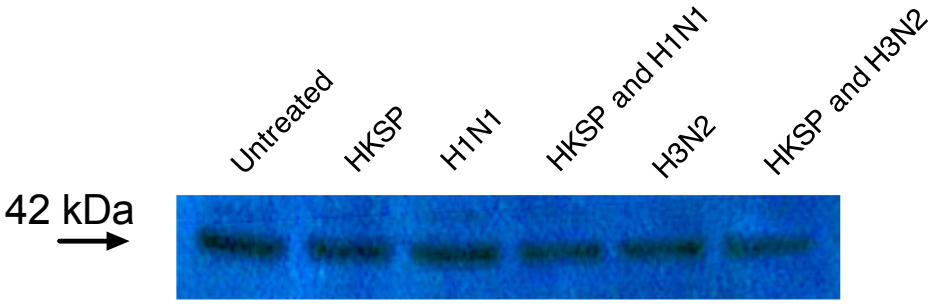

Figure 2.13 Housekeeping protein, β-Actin is detected in all cells. β-Actin protein produced by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP (or untreated as a control) was determined by western blot.

2.3.7 NEW STRAINS OF INFLUENZA A VIRUS INHIBIT ADAPTIVE TH17 AND TH1 RESPONSES TO STREPTOCOCCUS PNEUMONIAE

After pathogen recognition by the immune system, APCs present pathogen antigens to naïve T cells via their MHC class II molecules. The TCR on the naïve T cell recognises

the antigen, which leads to T cell clonal expansion and differentiation into Th effector cells such as Th1 and Th17 cells [116,137,138]. Th1 cells produce IFN-γ, which is a crucial cytokine involved in defence against microbial pathogens [287]. As mentioned previously Th17 cells are the main producers of IL-17, which is critical in the clearance of *S.p*. from the lung [88]. Here, it is demonstrated that IAV inhibits adaptive immune responses to *S.p*. in primary human immune cells. The inhibition has occurred in cytokines involved in both the Th1 and Th17 responses. Supernatants from treated co-cultures ($CD14^+$ APCs and $CD3^+$ T cells) were used to detect for cytokines using ELISA (as per Section 2.2.7). Significant inhibition of HKSP-induced IL-17A and IFN-γ (Figure 2.14 A and B) (n=3) by new strains of IAV occurred. This inhibition occurred in the absence of elevated anti-inflammatory cytokines, TGF-β and IL-10 (Figure 2.15 A and B) (n=3).

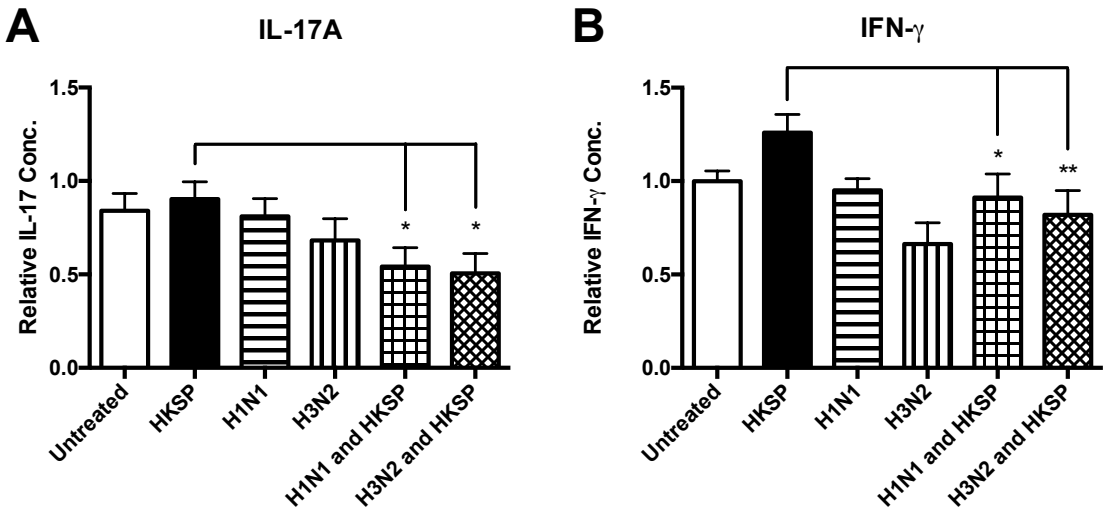

Figure 2.14 Live H1N1 and H3N2 infection inhibits HKSP-induced IL-17A and IFN-γ. The levels of (A) IL-17A and (B) IFN-γ secreted by $CD14^+$ APC and $CD3^+$ T cell co-cultures following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP alone versus cells treated with HKSP in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*p<0.05, **p<0.01).

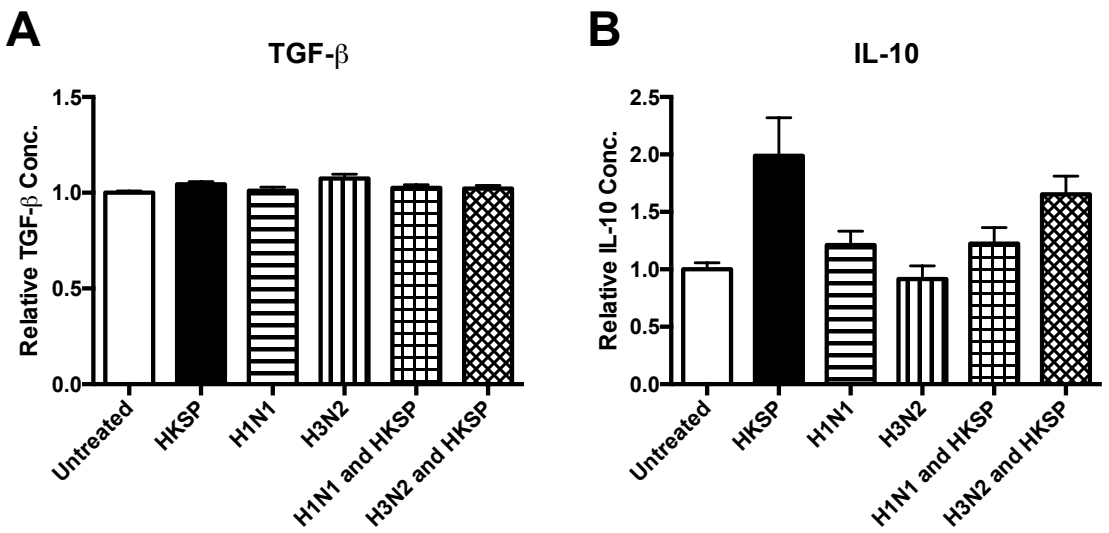

Figure 2.15 Levels of TGF-β and IL-10 do not increase during live IAV infection. The levels of (A) TGF-β and (B) IL-10 secreted by $CD14^+$ APC and $CD3^+$ T cell co-cultures following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP alone versus cells treated with HKSP in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

2.3.8 NEW BATCHES AND CLINICAL ISOLATES OF INFLUENZA A VIRUS STRAINS HAVE SIMILAR IMMUNOSUPPRESSIVE EFFECTS TO PREVIOUS ISOLATES ON ADAPTIVE RESPONSES

Previous studies carried out in the Viral Immunology Lab demonstrated that IAV inhibited *S.p.* driven adaptive cytokines secreted by human APC-T cells [350]. Upon receipt of new strains of live H1N1 and H3N2 from the NIBSC, previous studies were repeated to further develop and characterise the *ex vivo* human immune model for adaptive responses and to determine if any differences occurred (Table 2.9). The new results obtained for IL-17A, IFN-γ, TGF-β and IL-10 were in keeping with what was previously obtained for IAV.

Table 2.9 Comparison between inhibition of previous results against current results generated with new batches of H1N1 and clinical isolates of H3N2

Cytokine	H1N1		H3N2	
	Previous*	New**	Previous*	New**
IL-17A	0.25	0.36	0.56	0.39
IFN-γ	0.86	0.35	1.23	0.44
TGF-β	0.13	0.01	0.06	0.02
IL-10	-0.82	0.77	0.19	0.33

Yellow indicates inhibition of HKSP by IAV, whereas blue indicates no inhibition. Numerical values represent the difference between HKSP relative concentrations and HKSP and IAV relative concentrations. Negative numbers indicate that co-treated cells induced higher amounts of cytokine than HKSP alone treated cells. *n=9; **n=5.

2.4 DISCUSSION

T helper 17 cells have been identified as critical in the effective clearance of extracellular bacteria from the lung, particularly *S.p.* [88]. The majority of the research performed in this field is carried out in animal models [102,316–319]. However, the suitability of these models is questionable [320–322,327]. As many of the results obtained from animal models, are not reproduced in any human model, the viral immunology group in DCU developed an *ex vivo* human immune model [333,340,341], which was subsequently used to model influenza and *S.p.* co-infections [350]. This research demonstrated that IAV inhibited key pneumococcus driven innate cytokines secreted by human APCs and adaptive cytokines by T cells [350]. This study was performed with a lab-adapted virus and a clinical isolate of IAV and in-house generated HKSP. As new batches of H1N1, a new clinical isolate of H3N2, and commercially bought HKSP were being used for subsequent studies, additional characterisations of these was carried out. This was both to further develop the human model, but also to ensure that similar levels of immune induction/inhibition by new batches and clinical isolates of HKSP and IAV, respectively was occurring. As these studies use blood donations from healthy human donors, variability of immune responses was an initial concern due to donors being constantly exposed to a variety of pathogens. To combat this potential variability, a very high number of donors were used (n=20). This ensured that the new HKSP/IAV was being characterised in a robust manner and that accurate development of the model occurred, leading to a more complete indication of immune responses.

To initially ensure that the new strains of IAV were infecting isolated $CD14^+$ APCs, qPCR was used to detect for both H1N1 and H3N2 NP expression. As NP is expressed intracellularly during IAV infection, it serves as a reliable indicator to confirm IAV infection [340,352]. H1N1 and H3N2 NP was not detected in untreated cells, as expected. H1N1 NP was detected in all H1N1-infected cells, and H3N2 NP was detected in all H3N2-infected cells, with very little variability between donors. Expression of H3N2 NP was lower than H1N1 NP. This is most likely due to H1N1 being a laboratory-generated virus, whereas H3N2 is a clinical isolate. Levels of infectivity across donors was very reproducible, indicating that the ability of the viruses to infect APCs is not variable. It can be concluded that the inter-batch expression of the viral protein, NP, is very consistent.

To compare induction of immune responses by a new commercially bought HKSP to previously in-house generated HKSP, relative concentrations of key innate cytokines in response to the old and new batches were compared to one another (Table 2.7). Induction of IL-23, IL-6, and TGF-β was very similar between batches of HKSP. Induction of IL-27, IL-10, IL-1β, and IL-12p70 was not as strong in response to the new batch of

HKSP compared to the previous batch. However, despite this, induction of these cytokines was still strong.

Important innate Th17 polarising cytokines were studied, along with a Th1 polarising cytokine (IL-12p70). Anti-inflammatory cytokines, which maintain the balance between Th17 cells and T_{regs} were also examined as they can inhibit Th17 responses [241,246,248,249,356]. Interleukin-23 (IL-23), IL-6, IL-1β, and TGF-β are essential in Th17 responses to *S.p.*, however, TGF-β is also involved in anti-inflammatory responses via induction of T_{reg} cells which can inhibit Th17 responses as part of cell regulation and maintenance of homeostasis [176,353]. IL-12p70 is involved in the Th1 response through the production of IFN-γ, about which there is conflicting reports on its effect on the clearance of *S.p*. [3,185,293,294]. IL-27 has been implicated in both Th1 and Th17 responses [182,228,354]. IL-10 is an anti-inflammatory cytokine which can inhibit Th1 and Th17 responses [241,246,248,249,356].

Supernatants from immune cells were analysed using ELISA to determine the levels of secreted cytokines present. Influenza A Virus inhibited pneumococcus driven IL-23, IL-6, IL-27, and IL-12p70. Both H1N1 and H3N2 inhibited HKSP-induced IL-23, IL-6, and IL-27. Additionally, H1N1 inhibited HKSP-induced IL-12p70 and IL-10. The inhibition of IL-27 is of particular interest, due the complex modes of action of this cytokine. IL-27 is a multi-functional cytokine and depending on certain immune signals, its effects may differ greatly. The apparent contradiction of the role of IL-27 has been attributed to the ratio between the transcription factors; STAT1 and STAT3 [182]. IL-27 can induce robust levels of STAT1, which strongly inhibits the Th17 response, however this pathway requires IFNAR signalling. Indeed, in the absence of STAT1, IL-27 induces phosphorylated STAT3, which strongly activates the Th17 response [376]. Thus, the inhibition of these responses cannot be attributed to IL-27, as it itself has been inhibited directly by influenza. As mentioned above, IL-12p70 was only inhibited by the new batch of H1N1 and not by the new clinical isolate of H3N2, which differs from previous results. This discrepancy may actually be due to differences between the level of induction of IL-12p70 by new HKSP compared with previously used HKSP which was cultured in-house. Previous induction of IL-12p70 by HKSP (cultured in-house) was much higher than the induction by commercially bought HKSP.

Due to the anti-inflammatory effects of IL-10 and TGF-β, levels of these cytokines were analysed to determine a role, if any, in the inhibition of these innate cytokines. Elevated levels of IL-10 and TGF-β were not detected in treated samples, therefore the inhibition of the Th17 cytokines could not be due to the anti-inflammatory effects of these cytokines. Indeed, with the new viral stocks, an inhibition of HKSP-induced IL-10 by

H1N1 occurred. These results were compared to previous studies which had been carried out by other personnel in the lab (Table 2.8). Previous results demonstrated that IAV inhibited HKSP-induced IL-6, IL-23, IL-27, and IL-12p70, but did not inhibit IL-1β, IL-10 or TGF-β [350]. In keeping with previous results, pneumococcus-driven IL-23 and IL-27 were inhibited by new H1N1 and H3N2 and pneumococcus-driven IL-6 was inhibited by H3N2. However, the new batch of H1N1 also inhibited pneumococcus-driven IL-6 and IL-10. A reason for this may simply be that certain batches of influenza are more immunosuppressive than others, resulting in inhibition of a larger selection of cytokines. Overall, the conclusions of these studies are consistent with previous results that IAV inhibits early Th17 polarising cytokines in human APCs.

To examine the level of apoptosis across different treatments in our human *ex vivo* model, an apoptosis study was performed using flow cytometry. The level of apoptosis was not increased in cells co-treated with HKSP and IAV (Figure 2.11). The level of apoptosis was lower in these cells than in cells treated with HKSP alone. As cells treated with HKSP alone produce an abundance of cytokines, yet have a higher level of apoptosis, it can be deduced that apoptosis is not causing the inhibition of cytokines in cells-co-treated with HKSP and IAV. The level of apoptosis in HKSP-alone treated cells seemed curious. However, these cells strongly induce IL-23 and IL-1β and studies have shown that elevated levels of IL-23 and IL-1β can induce apoptosis [377–379]. This may be to prevent pathogenic effects caused by these cytokines. Additionally, to rule out protein synthesis shutdown, a western blot was performed, which determined that β-Actin protein production was stable across treatments, indicating that protein synthesis shutdown did not occur (Figure 2.12). As inhibition cannot be attributed to either anti-inflammatory effects of IL-10 and TGF-β, apoptosis, or protein synthesis shutdown, this may point to IAV directly targeting the innate Th17 pathway.

Innate immune cells can prime T cells to induce long-term immunity [116,137]. Recognition of antigens by T cells leads to T cell clonal expansion and differentiation into effector T cells such as Th or T_{reg} cells, depending on which cytokines are produced. Th cells include Th1 cells and Th17 cells, which produce their own range of cytokines, including IFN-γ and IL-17, respectively [116,137–142]. Both IL-17A and IFN-γ were studied due to their importance in the adaptive Th17 and Th1 responses to bacteria. The anti-inflammatory cytokines (TGF-β and IL-10) were also studied. Supernatants from immune cells were analysed using ELISA to determine the levels of secreted cytokines present. Both H1N1 and H3N2 inhibited HKSP-driven IL-17A and IFN-γ. There were no significant differences between treatments for TGF-β and IL-10, therefore inhibition of IL-17A and IFN-γ cannot be attributed to anti-inflammatory effects of these cytokines. The

adaptive results were compared to previous studies, which had been carried out with different batches and clinical isolates of IAV (Table 2.9). Previous results demonstrated that IAV inhibited IL-17A and IFNγ, but did not affect TGF-β or IL-10 [350]. Corroborating previous work, HKSP-driven IL-17A and IFN-γ were inhibited by new batches and clinical isolates of IAV, but neither TGF-β or IL-10 were affected.

Overall, the conclusions of these studies are consistent with previous results that IAV inhibits innate responses that drive Th17 and Th1 polarisation, which results in inhibition of adaptive Th17 and Th1 responses by human APCs, and by APC-T cell co-cultures. This points to IAV directly affecting early immune responses and is an important result especially due to the nature of the human model being employed. It also leads to important questions as to how the virus is inhibiting these responses.

3.0 MECHANISMS OF IAV INHIBITION OF TH17 AND TH1 IMMUNE RESPONSES IN HUMAN ANTIGEN PRESENTING CELLS

3.1 INTRODUCTION

We have demonstrated that IAV inhibits *S.p.* induced innate and adaptive cytokines in human APCs and T cells, respectively. The cytokines which have been inhibited are involved in the Th17 and Th1 immune responses, which are effective in the clearance of bacterial infections. This inhibition was shown to not be due to the indirect effects of the anti-inflammatory cytokines IL-10 and TGF-β or due to apoptosis. However, there are other effects (both direct and indirect) of IAV infection that may have been responsible for the immune inhibition.

During viral infection, cells such as macrophages and DCs which differentiate from monocytes and are involved in the innate immune response secrete large quantities of IFNs, which play a pivotal role in anti-viral responses against influenza infection [149–156]. However, the role type I IFNs play on immune responses to *S.p.* have been conflicting, with different studies claiming either protective or detrimental effects due to their presence. As we have demonstrated early inhibition of *S.p.* immune responses by IAV, the possibility that this inhibition may be due to type I IFNs had to be explored, as it was unknown whether type I IFN would be induced at such an early stage in our *ex vivo* human immune model. Type I IFNs include the subtypes IFN-α and IFN-β. In humans, there are thirteen distinct IFN-α proteins and a single IFN-β protein [157]. Type I IFNs signal through a shared receptor; IFNAR, signalling through which triggers downstream anti-viral responses [2]. Type I IFN induction is modulated by IRF3 and IRF7 [158–160]. IRF3 contributes to early IFN-β production, which triggers synthesis of IRF7. Production of IRF7 leads to an increase in IFN-β expression, resulting in IFN-α production in a "positive amplification loop" [163,164]. As mentioned previously, some studies have shown that type I IFNs inhibit Th17 responses in humans and mice [2,3,380,381]. One study found that without functional IFNAR signalling, mice were more resistant to secondary bacterial pneumonia post-influenza infection than mice with functional IFNAR signalling [2]. For many years, IFN-β has even been used as a treatment for MS due to its ability to inhibit Th17 responses [380,381]. However, contrary to these studies, it has also been shown that IFN-α expression prior to respiratory infection with *S.p*. improved the outcome of pneumococcal infection in mice, and that IFNAR signalling can be crucial

for *S.p*. bacterial clearance in mice [288–292]. Additionally, the influenza viral protein NS1 is known to inhibit type I IFNs [38,54]. There have been a significant number of studies carried out in animal models, however there is a scarcity of studies in human models, therefore there is a need to study this further in order to fully explore this complex relationship.

To address whether type I IFNs were involved in the indirect inhibition of Th17 responses in our *ex vivo* human model, we sought to determine the expression levels of type I IFN mRNA and protein. An extensive examination of mRNA via quantitative polymerase chain reaction (qPCR) was performed. The experimental procedure was designed to ensure that all of the subtypes were able to be detected. To detect for type I IFN protein, ELISA was performed to detect for the presence of IFN-α and IFN-β protein.

Due to the conflicting reports surrounding the role of type I IFNs in *S.p.* infections, we also sought to determine other possible reasons to explain the early inhibition of *S.p.* responses by IAV. This led us to examine the normal immune response to *S.p.*, with a view to identifying aspects of immunity that IAV could be targeting.

Once a pathogen enters the host cell, it is recognised by innate receptors such at TLRs on immune cells or on infected cells, which trigger signalling cascades resulting in the production of effector molecules such as cytokines which protect against the invading pathogen [115,116]. Once the pathogen is sensed by receptors such as TLRs on or within APCs, the APCs become activated, secrete distinct cytokines, and present antigens to naïve T cells, which leads to T cell clonal expansion and differentiation into effector T cells such as Th17 cells. The initial innate sensing has a profound effect on the type of adaptive response which is subsequently elicited [116,137,138].

Toll-Like Receptors are involved in sensing pathogens such as *S.p.* and IAV, and TLR signalling helps the immune system mount appropriate responses. There are ten different types of TLRs in humans (TLR1-TLR10), with each responding to an array of PAMPs from a variety of microbes. TLR 1, 2, 4, 5, and 6 are expressed on the cell surface, whereas TLR 3, 7, 8 and 9 are expressed intracellularly [121,122]. TLR2, TLR4, and TLR9 are the three TLRs which specifically sense various components of *S.p.* TLR2 recognises pneumococcal cell wall components such as lipoteichoic acid (LTA) and lipoproteins [123,124]. TLR4 recognises pneumolysin (PLY), and TLR9 recognises pneumococcal DNA comprising unmethylated CpG motifs [126–128,130].

It was hypothesised that IAV may be directly targeting individual *S.p.* associated TLRs (TLR2, TLR4, and TLR9). Therefore, to assess this, individual mono-agonists to these *S.p.* associated TLRs were carefully selected to activate specific TLR responses in our human model. Each agonist chosen is a mono-stimulant to ensure definitive stimulation of each TLR. Dose responses were initially performed to determine the optimum

concentration of each TLR agonist to use in further experiments. Once the optimum dose of each TLR agonist had been established, cells were infected with live IAV and stimulated with TLR agonists to determine if IAV had the ability to directly suppress TLR activation of immune responses. The supernatants from TLR stimulated cells were used for ELISA to detect for the presence of pneumococcus driven-cytokines which have been shown to be inhibited by IAV previously.

To complement this research, we also sought to determine if IAV was directly targeting the transcription factor, RORC. RORC has a very specific role in the development of Th17 cells and IL-17 production, but also plays a role in the upregulation of IL-23 expression [187,190–193,196–201]. To determine possible mechanisms whereby IAV is targeting immune pathways, the effect of IAV on TLR-induction of RORC was examined. RORC was of interest due to its unique role in Th17 differentiation.

Additionally, recent research has revealed the effects of TLR5 signalling (which is not associated with *S.p.* detection) on bacterial infections, suggesting that it may induce protective responses [382–384]. Furthermore, administration of TLR5 agonists elicited protection in mice against a range of bacterial infections such as *Clostridium difficile*, vancomycin-resistant *Enterococcus*, and *S.p.* [385–387]. Most recently, it has been shown that a TLR5 agonist has improved the efficacy of antibiotics in treating IAV and *S.p.* co-infections in mice [388], although the mechanisms by which TLR5 signalling induce these responses is not known. As there is currently a lack of research being performed in human models, we have therefore sought to establish if a TLR5 agonist can be used in our human model to restore previously observed inhibition of *S.p*-induced cytokines by IAV [350].

The findings presented in this chapter are entirely novel. We have demonstrated that IAV infection inhibition of Th17 and Th1 polarising cytokines are not due to anti-viral type I IFNs. Additionally, this is the first study to examine the effect of live IAV on TLR induction of cytokines secreted by human APCs, with additional research performed on RORC, of which largely only the mouse variant ROR-γ has been studied.

Specific aims of this chapter were:

- Determine expression levels of type I IFN mRNA and protein in our *ex vivo* human model using qPCR and ELISA, respectively,
- Characterise immune responses to TLR agonists by performing dose-response analyses to establish appropriate concentrations of each TLR agonist used,
- Determine if IAV affects specific TLR-activation of immune responses,
- Examine the effect of IAV on TLR-induced RORC, and

- Determine if a TLR5 agonist may be used to restore IAV-inhibited immune responses to HKSP.

3.2 MATERIALS AND METHODS

Table 3.1 List of additional reagents to Table 2.1 (Chapter 2) used in this study

Product	Catalogue Number	Company
Poly(I:C) HMW	tlrl-pic	Invivogen, Toulouse, France
LyoVec transfection reagent	lyec-1	
Lipoteichoic acid from *Staphylococcus aureus* (LTA-SA)	tlrl-slta	
Ultra-pure Lipopolysaccharide from *E. coli* (LPS-EB)	tlrl-3pelps	
Class A CpG oligonucleotide (ODN 2216)	tlrl-2216-1	
Flagellin from *Salmonella typhimurium* (FLA-ST)	tlrl-stfla	
Human IFN-β DuoSet ELISA	DY814	R&D Systems, UK
Human Interferon-α ELISA	41100-1	
Realtime Ready Catalog Assay - IFNA2 Human Gene	145795	Roche Diagnostics, UK
Realtime Ready Catalog Assay - IFNB Human Gene	145797	
Realtime Ready Catalog Assay – RORC Human Gene	102571	
UPL Human GAPD Gene Assay	5190541001	Sigma Aldrich, Wicklow, Ireland

3.2.1 ISOLATION OF PRIMARY HUMAN IMMUNE CELLS

Human PBMCs were isolated from healthy human donors as per Section 2.2.1. and cell yield was determined as per Section 2.2.2.

3.2.2 SEPARATION OF CD14$^+$ CELLS FROM PBMCs USING MICROBEAD SEPARATION

CD14$^+$ cells were purified from PBMCs as per Section 2.2.3.

3.2.3 VIRUS INFECTION OF CD14$^+$ ANTIGEN PRESENTING CELLS

Viral infection of CD14$^+$ APCs was performed as per Section 2.2.4.

3.2.4 STREPTOCOCCUS PNEUMONIAE STIMULATION OF CD14$^+$ ANTIGEN PRESENTING CELLS

CD14$^+$ APCs were exposed to HKSP as per Section 2.2.5.

3.2.5 POLY(I:C) TRANSFECTIONS

3.2.5.1 Background

Transfection is a technique whereby nucleic acids are delivered directly into the cytoplasm by non-viral methods. Transfection can be performed using chemical reagents, cationic lipids, and physical methods. Cationic lipids work as the cationic head of the lipid associates with negatively charged phosphates on the nucleic acid. The phospholipid structure encourages fusion with negatively-charged cellular membranes which aid in delivery of the nucleic acid into the cell [389,390]. Poly(I:C) is a TLR3 agonist and due to the intracellular location of TLR3, transfection was performed. Poly(I:C) was used as a suitable positive control for experiments concerning IFN-α and IFN-β as Poly(I:C) is known to induce robust, early, interferon responses.

3.2.5.2 Method

Poly(I:C) at a concentration of 1 µg/ml, was mixed with 100 µl LyoVec^{TM} solution and incubated at RT for at least 15 minutes. Isolated CD14$^+$ APCs at a density of 10^6 cells/ml were stimulated with the Poly(I:C) and LyoVec^{TM} solution (Invivogen) and incubated for 24 h at 37°C.

3.2.6 TOLL LIKE RECEPTOR AGONIST STIMULATIONS

Lipoteichoic acid from *Staphylococcus aureus* (LTA-SA) (Invivogen) was used as a TLR2 agonist at a range of doses from 15 µg to 40 µg for initial dose response experiments. Ultra-pure Lipopolysaccharide from *E. coli* (LPS-EB) (Invivogen) was used a TLR4 agonist at a range of doses from 50 ng to 250 ng. Ultra-pure LPS-EB was chosen to ensure that no stimulation of TLR2 also occurred. Class A CpG oligonucleotide (ODN 2216) was used as a TLR9 agonist at a concentration of 2 µM to determine if immune responses could be induced. Flagellin from *Salmonella typhimurium* (FLA-ST) was used as TLR5 agonist at 100 ng and 200 ng. Dose responses were performed with the various

doses of each agonist to determine optimal concentrations. A final concentration of 20 μg of LTA-SA was chosen as the optimal concentration. A final concentration of 100 ng of LPS-EB was chosen as the optimal concentration. A concentration of 2 μM of ODN 2216 was used in all experiments. A final concentration of 100 ng of FLA-ST was chosen as the optimal concentration. Isolated $CD14^+$ cells at a density of 10^6 cells/ml were stimulated with various agonists at chosen doses and incubated for 24 h at 37°C.

3.2.7 BIOINFORMATICS

Thirteen human IFN-α mRNA sequences, corresponding to each subtype were retrieved from Pubmed [391]. Each sequence was compared to one another using BLAST to identify homologous sequences (IFNA1 – NM_024013.2; IFNA2 – NM_000605.3; IFNA4 – NM_021068.2; IFNA5 – NM_002169.2; IFNA6 – NM_021002.2; IFNA7 – NM_021057.2; IFNA8 – NM_002170.3; IFNA10 – NM_002171.2; IFNA13 – NM_006900.3; IFNA14 – NM_002172.2; IFNA16 – NM_002173.3; IFNA17 – NM_021268.2; IFNA21 – NM_002175.2). Homologous sequences were used to ensure any primers/probes targeted every subtype to aid in downstream applications.

3.2.8 REAL TIME POLYMERASE CHAIN REACTION (qPCR)

cDNA samples were amplified as per Section 2.2.8 with deviations. Expression of IFN-α (Assay ID: 145795), IFN-β (Assay ID: 145797), and RORC (Assay ID: 102571) was normalised to the expression of the reference gene, GAPDH. The chosen IFN-α assay detected a sequence which is common to all IFN-α subtypes. The chosen RORC assay detected both variants (ROR-γ and ROR-γt).

A 20 μl volume reaction was set up for each sample using 2-8 μl of cDNA as a template. The reaction was set up as follows:

Table 3.2 Reagents and volumes used in preparation of qPCR experiment

Reagent	Volume (μl)
Probes Master Mix	10.0
Gene of Interest Probe/Primer Mix	2.0
Reference Gene Primer	0.5
Reference Gene Probe	0.5
cDNA	X
PCR Grade Water	X

The following run settings were set up on the Lightcycler Nano:

1. Hold at 95°C for 600 seconds (ramp 4.4°C/s or 4.0°C/s)
2. Two-step amplification repeated for 50 cycles:
    a. 95°C for 10/20 seconds (ramp 4.4°C/s or 5.0°C/s)
    b. 60°C for 30/40 seconds (ramp 2.2°C/s or 4.0°C/s)
3. Hold at 40°C for 30 seconds (ramp 2.2°C/s)

The fold change in gene expression was calculated using the equation: $2^{(-\Delta\Delta Cq)}$.

3.2.9 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Supernatant from treated cells was used to detect for the following cytokines using ELISA kits; IFN-α, IFN-β, IL-6, IL-1β, IL-27, TGF-β, IL-10 (R&D systems), IL-23, and IL-12p70 (Biosciences) according to manufacturer's protocol. Samples and standards were plated either in duplicate or triplicate to ensure accurate quantitative results were obtained.

3.2.10 STATISTICAL ANALYSIS

Statistical analyses were performed as per Section 2.2.11.

3.3 RESULTS

3.3.1 IFN-α AND IFN-β mRNA MESSAGE IS ABSENT OR WEAKLY EXPRESSED BY HUMAN ANTIGEN PRESENTING CELLS

Many previous studies in mice have shown that late type I IFNs are responsible for the inhibition of the essential Th17 response [2,3]. To investigate if type I IFNs were involved in the inhibition of the Th17 response in our human *ex vivo* model, we used qPCR to detect for the gene expression of the type I IFN subtypes: IFN-α and IFN-β. In humans, there are thirteen distinct IFN-α proteins and a single IFN-β protein [157], therefore extensive bioinformatics research was carried out (Section 3.2.7) to ensure that all 13 subtypes of IFN-α could be detected. Each IFN-α mRNA sequence was retrieved from Pubmed and compared to one another to identify a homologous sequence. Once identified, a probe/primer (Roche Diagnostics, 2016) was chosen which would amplify the homologous IFN-α sequence and the IFN-β sequence. Poly(I:C) was used as a positive control to validate the experiments as it is sensed by TLR3 and is a strong inducer of type I IFNs. Interferon-α mRNA was undetectable in 3 out of 4 donors, with very low levels detectable in just one donor compared to cells treated with Poly(I:C) (Figure 3.1 A). Interferon-β mRNA was detected at very low levels by all cell samples compared to the positive control in all donors (n=4) (Figure 3.1 B).

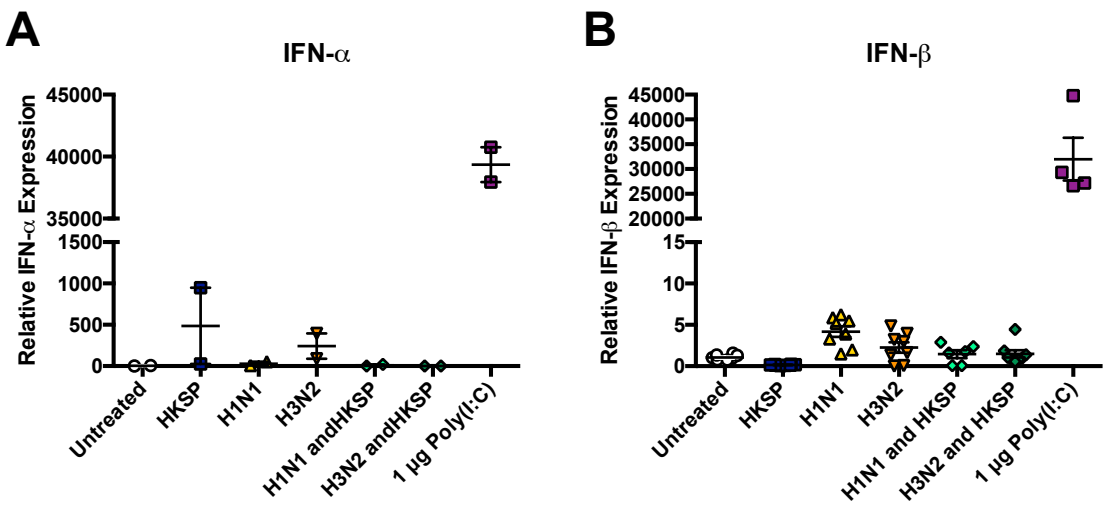

Figure 3.1 IFN-α and IFN-β are not strongly induced in treated samples. The levels of (A) IFN-α and (B) IFN-β mRNA expression by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated and 1 µg Poly(I:C) as controls were determined by qPCR. Each column represents mean amplification of genes of interest normalised to the mean expression of the reference gene, GAPDH (n=4). To show the amplification of IFN-α and IFN-β by cells other than the positive control, the x-axis was split into two sections with varying ranges.

3.3.2 IFN-α AND IFN-β PROTEIN IS WEAKLY INDUCED IN HUMAN ANTIGEN PRESENTING CELLS

As IFN-α and IFN-β mRNA was detected, albeit at a low level, the level of IFN-α and IFN-β protein expression was measured. To determine if type I IFN protein was being produced, ELISAs were performed to detect for the presence of IFN-α and IFN-β protein (Section 3.2.9). Neither IFN-α or IFN-β protein was strongly detected in any samples, apart from the Poly(I:C) treated samples (positive control), which induced a strong response (Figure 3.2). Very low levels of IFN-α protein was secreted by all cells, apart from those treated with HKSP, and co-treated with HKSP and H3N2, where none was detected (Figure 3.2 A). IFN-β was detectable in very low amounts in all cells and was induced strongly by Poly(I:C) stimulation (Figure 3.2 B).

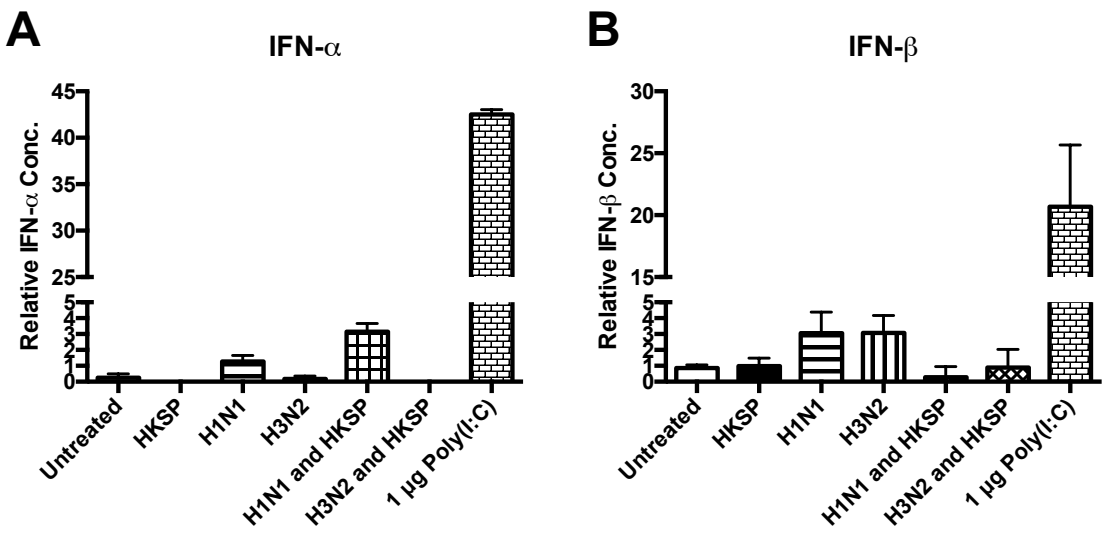

Figure 3.2 IFN-α and IFN-β are not strongly induced in treated samples. The levels of (A) IFN-α (n=3) and (B) IFN-β (n=4) secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 alone or in combination with HKSP or untreated and 1 µg Poly(I:C) as controls were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in the same donor.

3.3.3 INFLUENZA A VIRUS INFECTION SPECIFICALLY TARGETS TLR2 PATHWAY IN HUMAN ANTIGEN PRESENTING CELLS

It has been demonstrated in mice that in the absence of TLR2 signalling, transmission of *S.p.* occurred more efficiently during co-infection with influenza [316]. Both human and mouse models have shown that TLR2 senses *S.p.* infections [123,124,392]. TLR2 recognises pneumococcal cell wall components such as lipoteichoic acid (LTA) and lipoproteins [123,124]. To characterise the immune response to TLR2 agonism in our human *ex vivo* model, the optimum dose of the TLR2 agonist was determined by performing dose responses using a range of concentrations from 15 µg/ml to 30 µg/ml (Table 3.3 and Appendix 1.1). Other studies varied in concentrations used (1-100 µg/ml) [393–398]. Resulting from these assays, LTA-SA was used as a TLR2 agonist at a concentration of 20 µg/ml.

Table 3.3 Relative cytokine concentrations across different TLR2 agonist doses

Relative cytokine conc. (n=3)	TLR2 agonist Concentration (µg/ml)		
	15	**20**	**30**
IL-23	0.815	1.009	0.897
IL-6	0.968	0.958	0.932
IL-27	1.117	1.130	1.089

We sought to establish if IAV had an effect on TLR2 agonism in human APCs. We separated PBMCs of buffy coats from healthy human donors and have demonstrated that IAV infection inhibits LTA-SA induced responses in human APCs 24 hours post-infection. We found that the H1N1 subtype of IAV significantly inhibited LTA-SA induced Th17 polarising IL-23 (Figure 3.3 A), whereas both IAV subtypes of IAV significantly inhibited LTA-SA induced Th17 polarising TGF-β (Figure 3.3 B) (n=9). There were no significant effects on the Th17 polarising cytokines, IL-6 (Figure 3.4 A) and IL-1β (Figure 3.4 B), or on the multi-functional cytokine, IL-27 (Figure 3.4 C) (n=9). There was also no effect on the Th1 polarising cytokine, IL-12p70 (Figure 3.4 D) (n=5). There was no increase in the anti-inflammatory cytokine, IL-10 from cells co-treated with LTA-SA and IAV (Figure 3.5) (n=9).

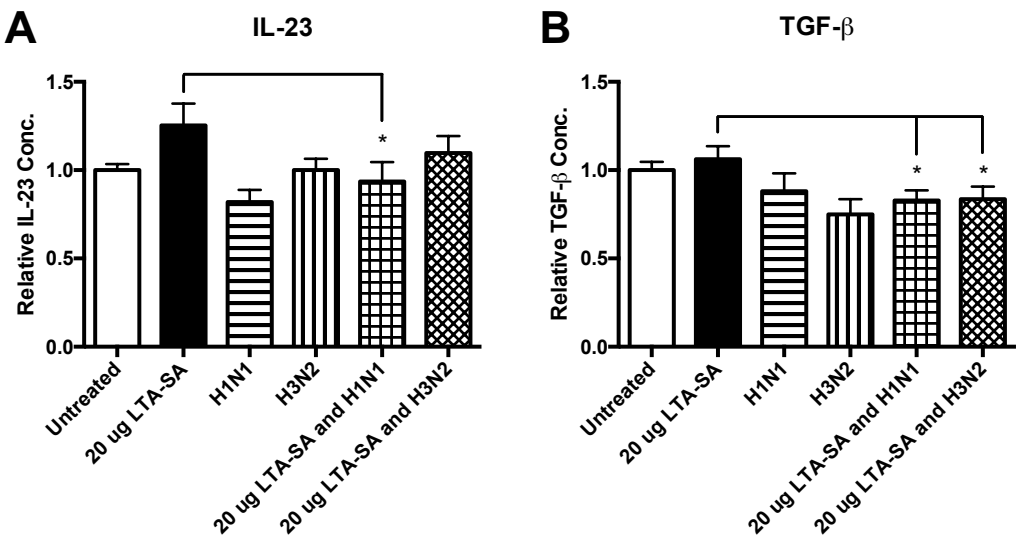

Figure 3.3 Live IAV infection inhibits LTA-SA-induced IL-23 and TGF-β. The levels of (A) IL-23 and (B) TGF-β secreted by $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), live H1N1 or H3N2 alone or in combination with LTA-SA or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 9 donors (n=9). Statistical analyses were performed to compare cytokine levels secreted by cells treated with LTA-SA alone versus cells treated with LTA-SA in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*$p<0.05$).

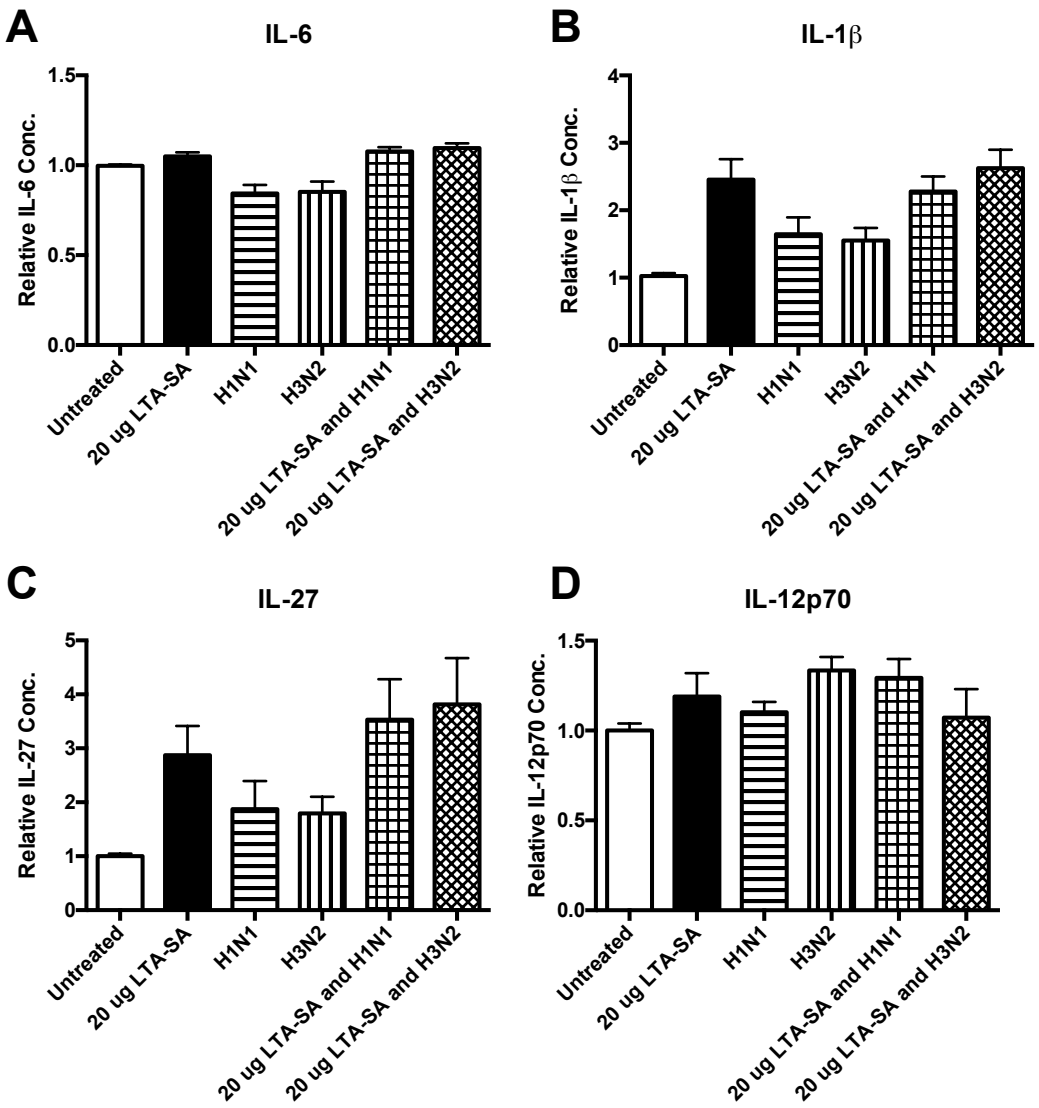

Figure 3.4 LTA-SA-induced IL-6, IL-1β, IL-27, and IL-12p70 are not inhibited by live H1N1 or H3N2 infection. The levels of (A) IL-6 (n=9), (B) IL-1β (n=9), (C) IL-27 (n=9), and (D) IL-12p70 (n=5) secreted by $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), live H1N1 or H3N2 alone or in combination with LTA-SA or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Statistical analyses were performed to compare cytokine levels secreted by cells treated with LTA-SA alone versus cells treated with LTA-SA in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

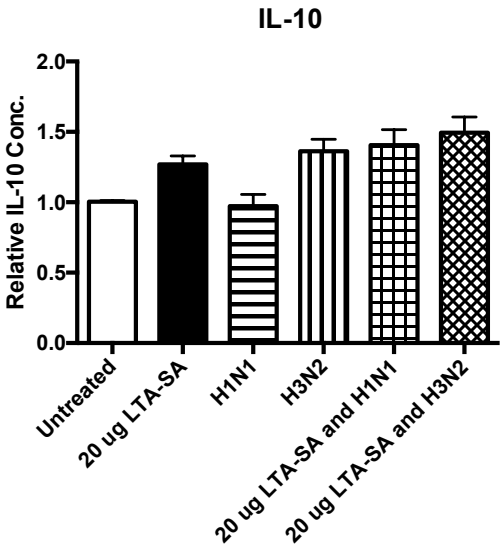

Figure 3.5 LTA-SA-induced IL-10 is not affected by live H1N1 or H3N2 infection. The levels of IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), live H1N1 or H3N2 alone or in combination with LTA-SA or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 9 donors (n=9). Statistical analyses were performed to compare cytokine levels secreted by cells treated with LTA-SA alone versus cells treated with LTA-SA in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

3.3.4 INFLUENZA A VIRUS INFECTION INHIBITS TLR4 AGONIST-INDUCED TGF-β IN HUMAN ANTIGEN PRESENTING CELLS

Studies have shown that TLR4 is triggered in response to *S.p.* infection by recognising PLY in both human cell lines and mice [126–128]. To characterise the immune response to TLR4 agonism in our human *ex vivo* model, the optimum dose of the TLR4 agonist was determined by performing dose responses using a range of concentrations from 50 ng/ml to 250 ng/ml (Table 3.4 and Appendix 1.2). Other studies varied in concentrations used (20-200 ng/ml) [399–402]. Resulting from these assays, LPS-EB was used as a TLR4 agonist at a concentration of 100 ng/ml.

Table 3.4 Relative cytokine concentrations across different TLR4 agonist doses

Relative cytokine conc. (n=3)	TLR4 agonist Concentration (ng/ml)			
	50	**100**	**150**	**250**
IL-23	6.829	7.974	7.693	4.771
IL-6	1.095	1.084	1.068	1.037
IL-27	2.888	4.626	2.646	2.179
IL-12p70	2.688	4.321	2.914	3.522

Cytokines involved in the Th17 response to *S.p.* infection were analysed to determine if IAV affected responses to LPS-EB, which is a TLR4 agonist. We demonstrated, using isolated PBMCs from healthy human donors that the TLR4 mono-agonist does not induce a very strong immune response and that IAV inhibits LPS-EB-induction of the multi-functional cytokine, TGF-β secreted by these cells (n=9) (Figure 3.6). LPS-EB induction of IL-12p70 was reduced by H3N2, although not significantly (n=5) (Figure 3.7 A). There was a slight increase in anti-inflammatory IL-10 (Figure 3.7 B) (n=9) from cells treated with LPS-EB and IAV, although this increase was not statistically significantly. LPS-EB induction of IL-23 (Figure 3.8 A), IL-6 (Figure 3.8 B), IL-1β (Figure 3.8 C), or IL-27 (Figure 3.8 D) were not inhibited by IAV.

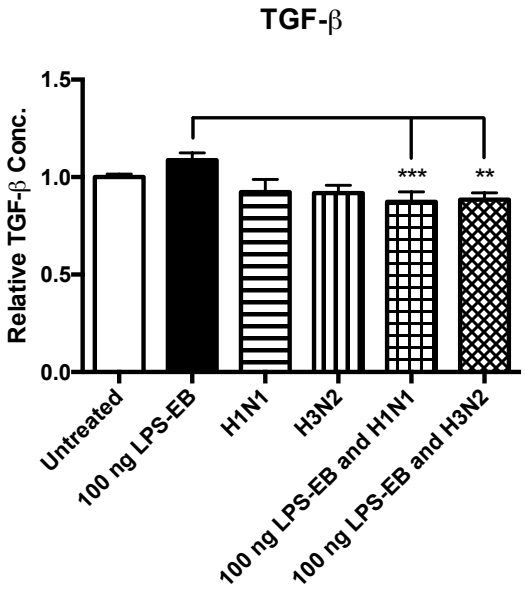

Figure 3.6 Live H1N1 and H3N2 infection inhibits LPS-EB-induced TGF-β. The levels of TGF-β secreted by $CD14^+$ APCs following 24 hr treatment with a TLR4 agonist (LPS-EB), live H1N1 or H3N2 alone or in combination with LPS-EB or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 9 donors (n=9). Statistical analyses were performed to compare cytokine levels secreted by cells treated with LPS-EB alone versus cells treated with LPS-EB in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (**p<0.01, ***p<0.001).

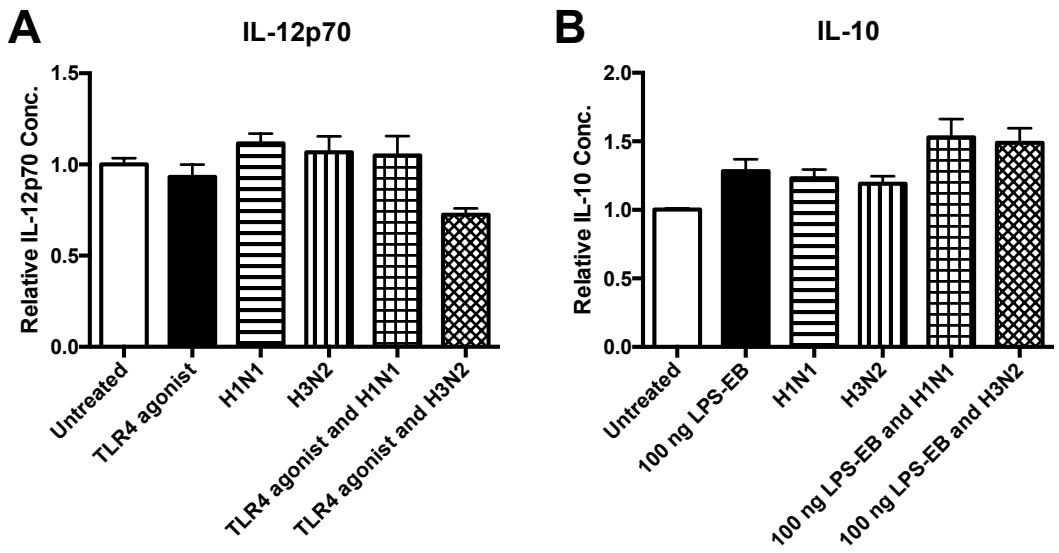

Figure 3.7 LPS-EB-induced IL-12p70 and IL-10 are not affected by live H1N1 or H3N2 infection. The levels of (A) IL-12p70 and (B) IL-10 secreted by $CD14^{+}$ APCs following 24 hr treatment with a TLR4 agonist (LPS-EB), live H1N1 or H3N2 alone or in combination with LPS-EB or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells treated with LPS-EB alone versus cells treated with LPS-EB in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

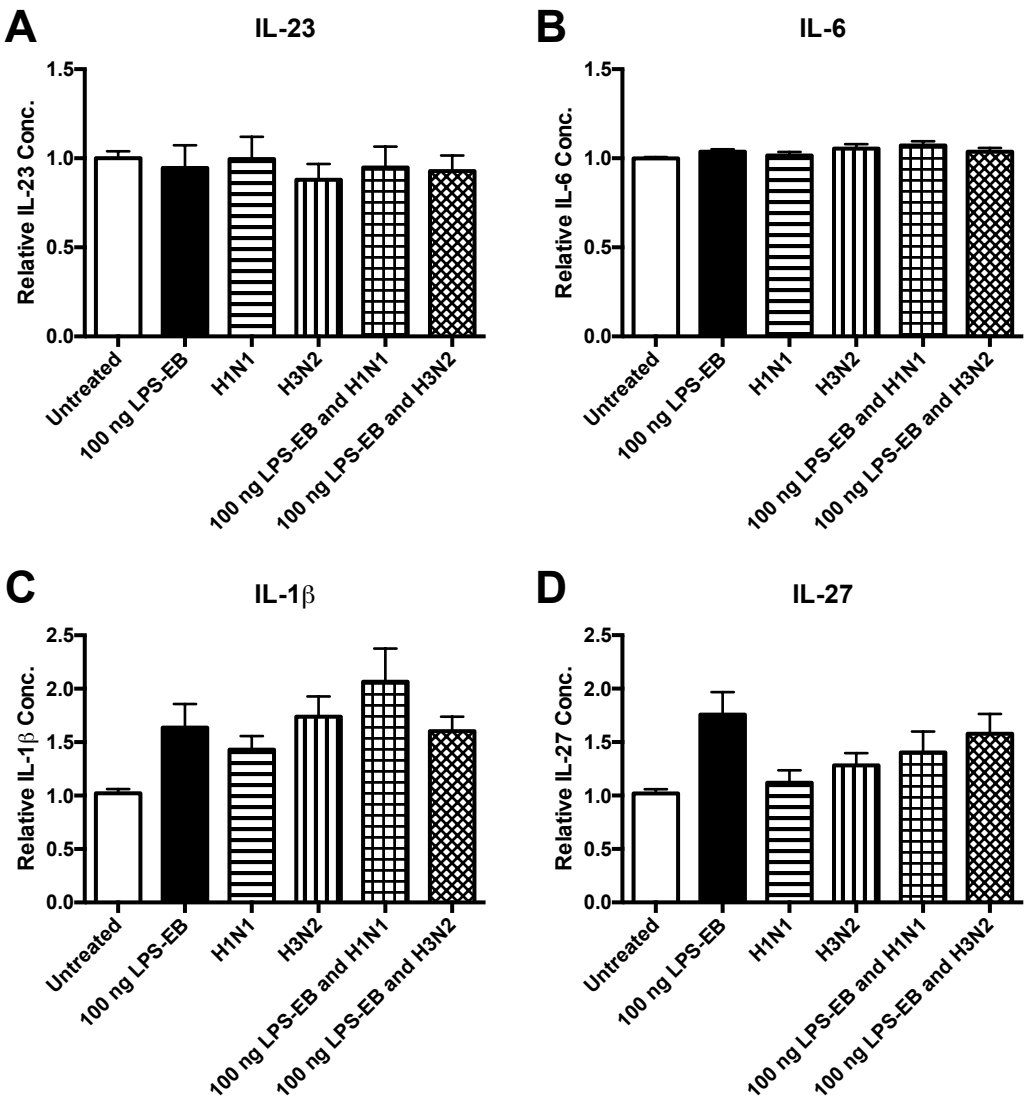

Figure 3.8 LPS-EB-induced IL-23, IL-6, IL-1β, and IL-27 are not inhibited by live H1N1 or H3N2 infection. The levels of (A) IL-23, (B) IL-6, (C) IL-1β, and (D) IL-27secreted by $CD14^+$ APCs following 24 hr treatment with a TLR4 agonist (LPS-EB), live H1N1 or H3N2 alone or in combination with LPS-EB or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 9 donors (n=9). Statistical analyses were performed to compare cytokine levels secreted by cells treated with LPS-EB alone versus cells treated with LPS-EB in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

3.3.5 INFLUENZA A VIRUS INFECTION SPECIFICALLY TARGETS TLR9 PATHWAY IN HUMAN ANTIGEN PRESENTING CELLS

It has previously been reported in mice that specific ligands for TLR9 protect against influenza, and that TLR9 plays a protective role in the early stages of *S.p.* infection [130,403]. To characterise the immune response to TLR9 agonism in our human *ex vivo*

model, the TLR9 mono-agonist (ODN 2216) was used to stimulate primary human immune cells at a concentration of 2 μM (Table 3.5 and Appendix 1.3). Other studies varied in concentrations (1-2.5 μM) [120,404–408]. Resulting from these assays, ODN 2216 was used as a TLR9 agonist at a concentration of 2 μM.

Table 3.5 Relative cytokine concentrations in response to TLR9 agonist

Relative cytokine conc. (n=3)	TLR9 agonist Concentration (μM)
	2
IL-23	7.139
IL-6	1.003
IL-27	1.206
IL-12p70	0.884

This TLR9 mono-agonist was used to stimulate primary human immune cells to determine if IAV affected TLR9 agonism. We demonstrated that IAV infection inhibited ODN 2216 induced responses from human APCs. Both H1N1 and H3N2 inhibited IL-23 (n=9) and TGF-β (n=9) (Figure 3.9 A and B). There was a reduction of ODN 2216-induced IL-27 by IAV (n=9) (Figure 3.10 A), although this was not statistically significant. There was no inhibition of ODN 2216 induced IL-6 (Figure 3.10 B) and IL-1β (Figure 3.10 C) (n=9). ODN 2216 induction of IL-12p70 was not inhibited by IAV (n=5) (Figure 3.10 D). There was no increase in the anti-inflammatory cytokine, IL-10 (n=9) (Figure 3.10 E).

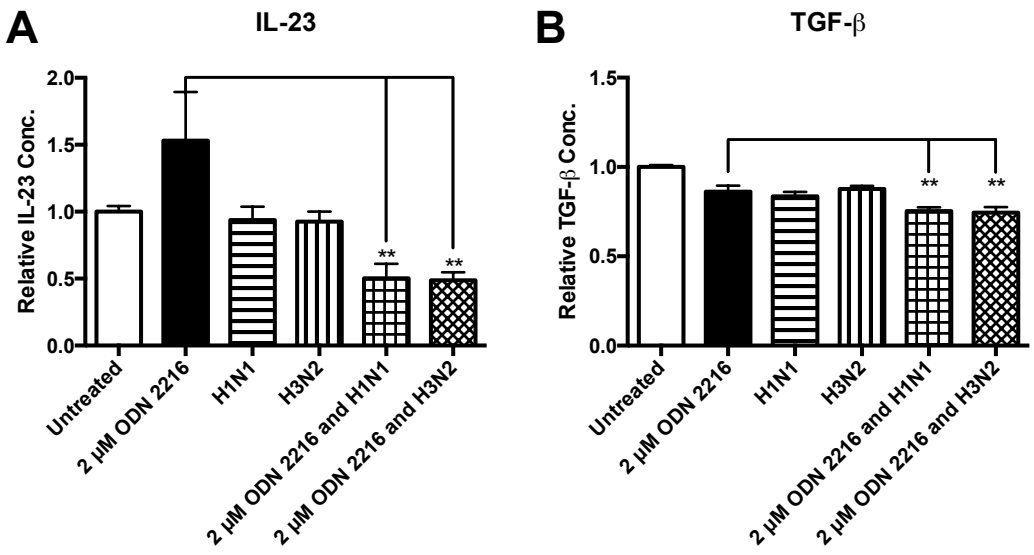

Figure 3.9 Live H1N1 and H3N2 infection inhibits ODN 2216-induced IL-23 and TGF-β. The levels of (A) IL-23 and (B) TGF-β secreted by $CD14^+$ APCs following 24 hr treatment with a TLR9 agonist (ODN 2216), live H1N1 or H3N2 alone or in combination with ODN 2216 or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 9 donors (n=9). Statistical analyses were performed to compare cytokine levels secreted by cells treated with ODN 2216 alone versus cells treated with ODN 2216 in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (**$p<0.01$).

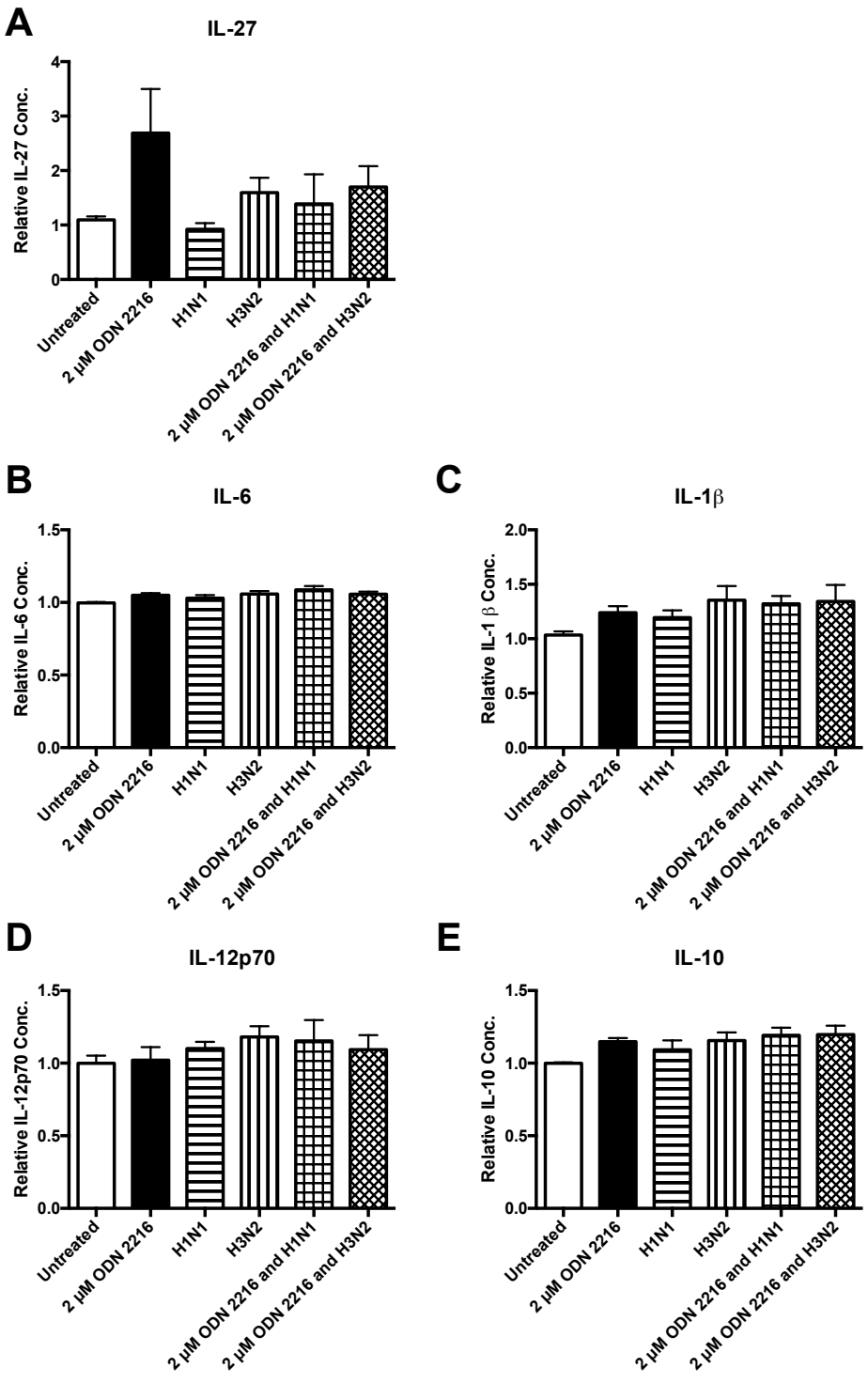

Figure 3.10 ODN 2216-induced IL-27, IL-6, IL-1β, IL-12p70, and IL-10 are not affected by live H1N1 or H3N2 infection. The levels of (A) IL-27 (n=9), (B) IL-6 (n=9), (C) IL-1β (n=9), (D) IL-12p70 (n=5), and (E) IL-10 (n=9) secreted by $CD14^+$ APCs following 24 hr treatment with a TLR9 agonist (ODN 2216), live H1N1 or H3N2 alone or in combination with ODN 2216 or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Statistical analyses were performed to compare cytokine levels secreted by cells treated with ODN 2216 alone versus cells treated with ODN 2216 in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

3.3.6 INFLUENZA A VIRUS INFECTION SPECIFICALLY TARGETS TLR9-INDUCTION OF RORC IN HUMAN ANTIGEN PRESENTING CELLS

RORC is a transcription factor specific to the Th17 response, which plays an important role in the upregulation of IL-23 expression [187,190–193,196–201]. As TLR2 agonist and TLR9 agonist induction of IL-23 has been inhibited by IAV (Figures 3.1 A and 3.9 A), we sought to investigate if this was due to IAV targeting expression of RORC in human APCs. The RORC gene encodes for two protein isoforms (RORγ and RORγt), therefore the assay chosen targeted both variants. We have determined that IAV does not inhibit TLR2-induction of RORC (Figure 3.11), however IAV does inhibit TLR9-induction of RORC (Figure 3.12).

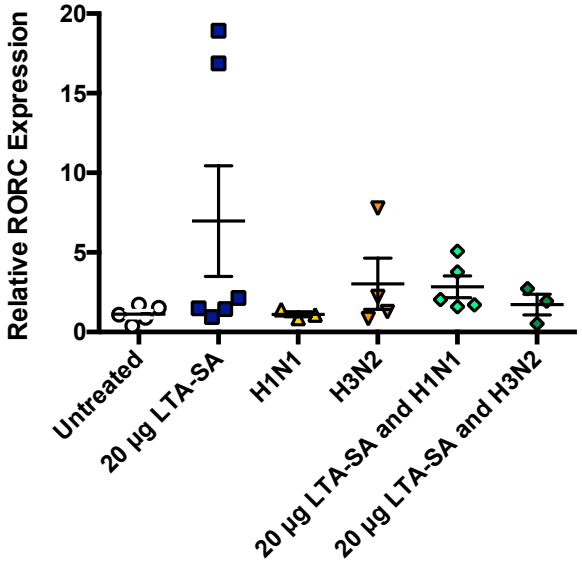

Figure 3.11 LTA-SA-induced RORC expression is not inhibited by live H1N1 or H3N2 infection. The levels of RORC mRNA expression in $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), live H1N1 or H3N2 alone or in combination with LTA-SA or untreated as a control were determined by qPCR. Each column represents mean amplification of gene of interest normalised to the mean expression of the reference gene, GAPDH (n=3). Statistical analyses were performed to compare RORC mRNA expression in cells treated with LTA-SA alone versus cells treated with LTA-SA in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

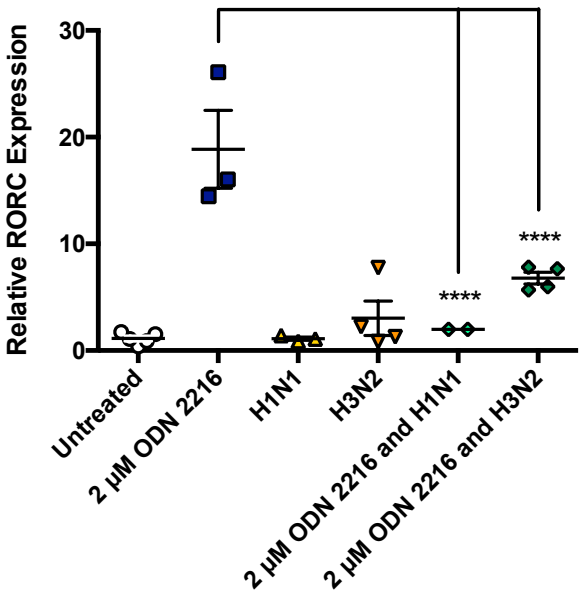

Figure 3.12 ODN 2216-induced RORC expression is inhibited by live IAV infection. The levels of RORC mRNA expression in $CD14^+$ APCs following 24 hr treatment with a TLR9 agonist (ODN 2216), live H1N1 or H3N2 alone or in combination with ODN 2216 or untreated as a control were determined by qPCR. Each column represents mean amplification of gene of interest normalised to the mean expression of the reference gene, GAPDH (n=3). Statistical analyses were performed to compare RORC mRNA expression in cells treated with ODN 2216 alone versus cells treated with ODN 2216 in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (****p<0.0001).

3.3.7 INFLUENZA A VIRUS INFECTION DOES NOT TARGET TLR5 PATHWAY IN HUMAN ANTIGEN PRESENTING CELLS

A TLR5 agonist has previously been shown to improve the efficacy of antibiotics in the treatment of IAV and *S.p* co-infections in mice [388]. To characterise the immune response to TLR5 agonism in our human *ex vivo* model, the optimum dose of the TLR5 agonist was determined by performing dose responses using concentrations of 100 ng/ml and 200 ng/ml (Table 3.6 and Appendix 1.4). Other studies varied in concentrations used (20-200 ng/ml) [395,409–411]. Resulting from dose response assays, FLA-ST was used as a TLR5 agonist at a concentration of 100 ng/ml.

Table 3.6 Relative cytokine concentrations across different TLR5 agonist doses

Relative cytokine conc. (n=3)	TLR5 agonist Concentration (ng/ml)	
	100	**200**
IL-23	2.268	1.933
IL-6	1.018	1.020
IL-27	0.804	0.759
IL-1β	1.246	1.208

The TLR5 agonist (FLA-ST) was used to stimulate cells and to determine if IAV had an effect on TLR5 agonsim in human APCs. We have demonstrated that IAV does not inhibit FLA-ST induced IL-23, TGF-β, IL-6, IL-1β, IL-27, IL-12p70, and IL-10 (Figure 3.13).

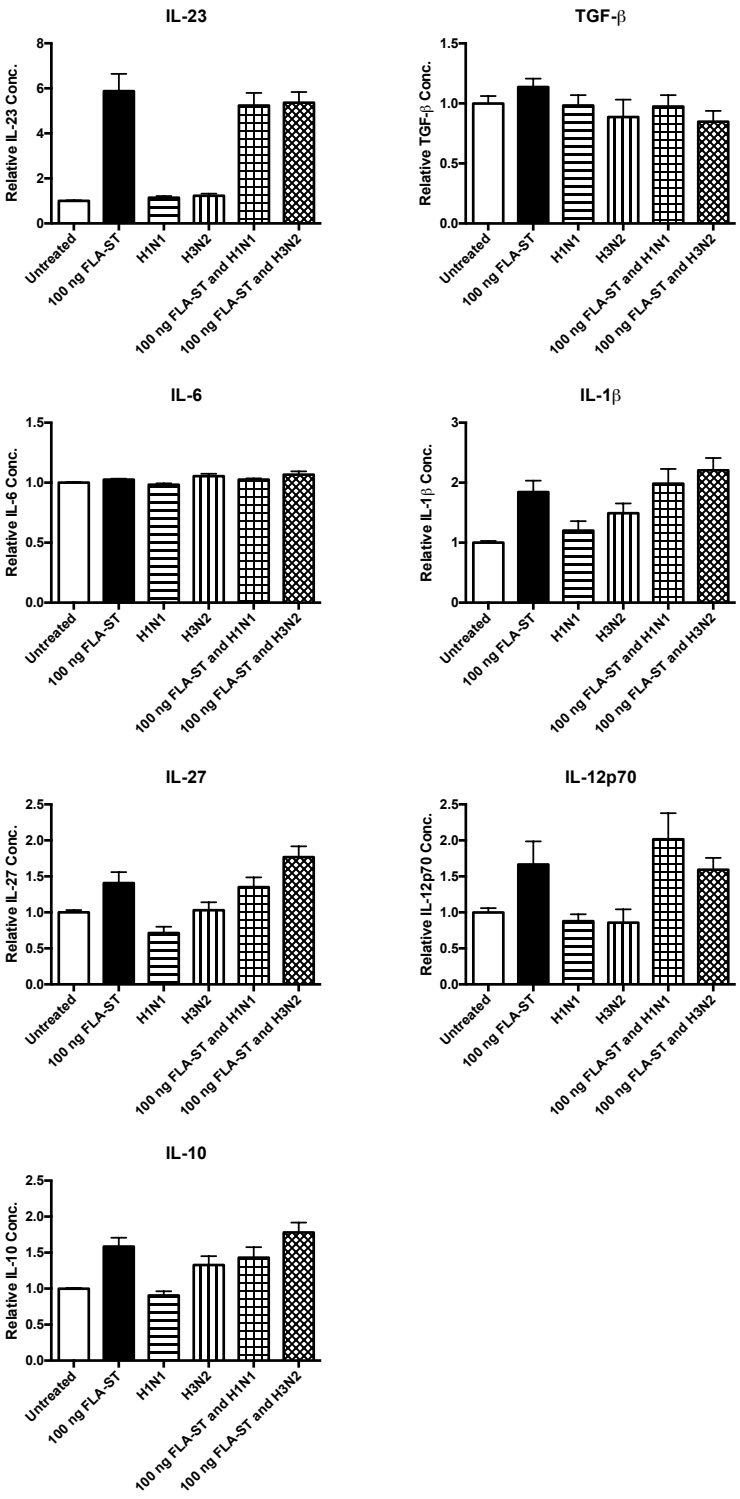

Figure 3.13 FLA-ST-induced cytokines are not inhibited by live IAV infection. The levels of IL-23, IL-6, IL-1β, IL-27, IL-12p70, and IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR5 agonist (FLA-ST), live H1N1 or H3N2 alone or in combination with FLA-ST or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells treated with FLA-ST alone versus cells treated with FLA-ST in combination with either live H1N1 or H3N2 by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

3.3.8 TREATMENT WITH A TLR5 AGONIST RESTORES INHIBITED IMMUNE RESPONSES TO HKSP DURING INFLUENZA INFECTION IN HUMAN ANTIGEN PRESENTING CELLS

Having established that TLR5 mono-agonism is not inhibited by IAV, we sought to establish if this TLR5 agonist could be used to restore previously observed inhibition of *S.p.*-induced cytokines by IAV [350]. We used a TLR5 agonist (FLA-ST) to stimulate cells to determine if IAV inhibition of HKSP could be circumvented in human APCs. We demonstrated that TLR5 stimulation restored IAV-inhibited HKSP-induced IL-23 (Figure 3.14 A) and IL-27 (Figure 3.14 B) (n=5). Stimulation with FLA-ST increased IL-12p70 levels to above those observed by HKSP treated cells alone (Figure 3.14 C) (n=5). TLR5 mono-agonist treatment did not have any effect on levels of IL-6 (Figure 3.15 A), IL-1β (Figure 3.15 B), TGF-β (Figure 3.15 C), and IL-10 (Figure 3.15 D) (n=5).

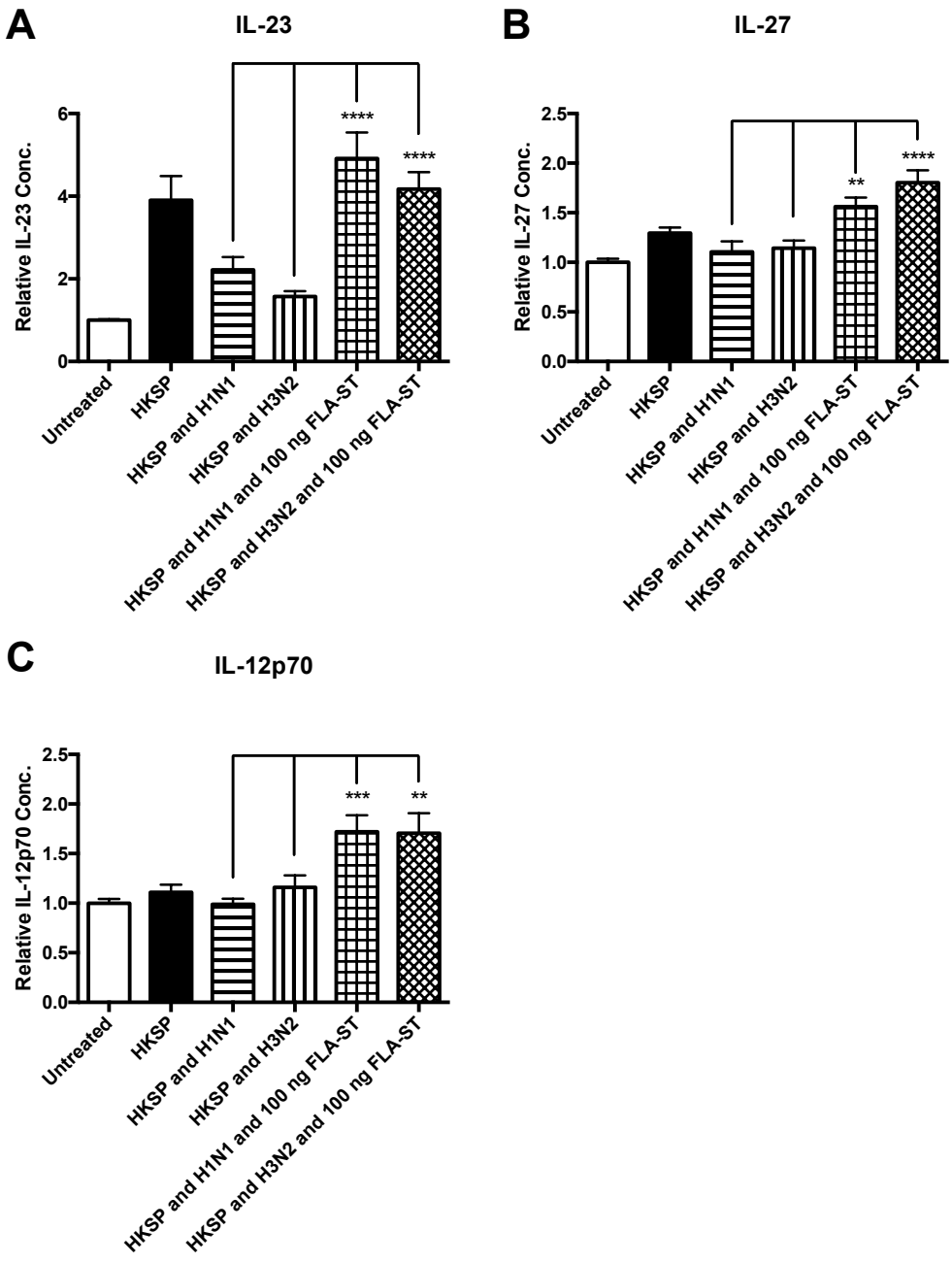

Figure 3.14 TLR5 agonism restores inhibited HKSP-induction of IL-23, IL-27, and IL-12p70 during IAV infection. The levels of (A) IL-23, (B) IL-27, and (C) IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 in combination with HKSP, live H1N1 or H3N2 in combination with HKSP and a TLR5 agonist (FLA-ST), or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP in combination with either live H1N1 or H3N2 versus cells treated with live H1N1 or H3N2 in combination with HKSP and FLA-ST by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (**p<0.01, ***p<0.001, ****p<0.0001).

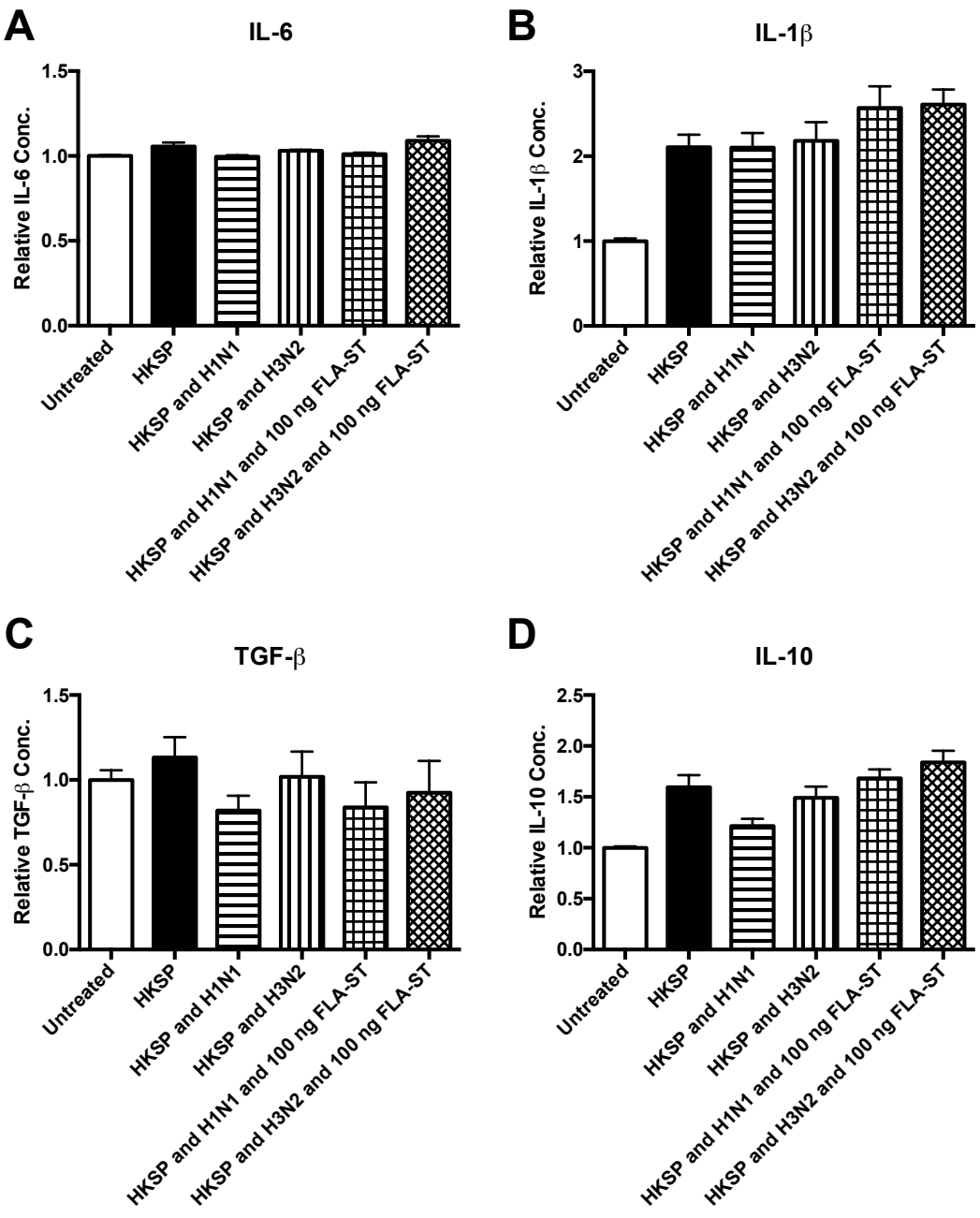

Figure 3.15 TLR5 agonism does not affect induction of IL-6, IL-1β, TGF-β, and IL-10 during HKSP-IAV co-infection. The levels of (A) IL-6, (B) IL-1β, (C) TGF-β, and (D) IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, live H1N1 or H3N2 in combination with HKSP, live H1N1 or H3N2 in combination with HKSP and a TLR5 agonist (FLA-ST), or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP in combination with either live H1N1 or H3N2 versus cells treated with live H1N1 or H3N2 in combination with HKSP and FLA-ST by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

3.4 DISCUSSION

There are conflicting reports surrounding type I IFNs and their role in the predisposition to secondary bacterial infections. It has been shown that type I IFNs induced by IAV infection inhibit Th17 responses in mice [2,3], however other studies have shown that IFN-α expression prior to respiratory infection with *S.p.* improved the outcome of pneumococcal infection in mice, and that functional IFNAR signalling can be crucial for *S.p.* clearance [288–292]. In addition, we have previously demonstrated that IAV infection of human APCs inhibits pneumococcus responses within 24 hours of co-infection/stimulation when type I IFNs are reported to be inhibited in mice [2,412]. This inhibition of responses to *S.p.* was shown to not be due to the anti-inflammatory effects of IL-10 and TGF-β. We sought to establish the levels of type I IFN mRNA message and protein induced in our *ex vivo* human model. To effectively answer the question of whether the immune inhibition occurs in the absence of type I IFNs in our human model, a robust experiment needed to be designed to detect all the species of type I IFN mRNA involved. Extracted RNA from treated samples was used to synthesise cDNA and subsequently perform qPCR. A probe and primers which amplified a sequence homologous to all 13 different human subtypes of IFN-α was chosen to ensure a definitive result.

In 3 out of 4 donors, IFN-α mRNA could not be detected, except by Poly(I:C) controls. In the one donor where mRNA was amplified, very low levels were detected, apart from the Poly(I:C) controls, which induced very strong expression of IFN-α. In cells co-treated with HKSP and IAV, IFN-α mRNA levels were lower than those in cells treated with HKSP alone. Very low IFN-β mRNA was detected by all cells, with robust induction by Poly(I:C). The levels of IFN-β mRNA induced by cells co-treated with HKSP and IAV was not significantly higher than those treated with HKSP alone. The inhibition cannot be attributed to IFN-α expression as minor amplification only occurred in one donor and that amplification was extremely low by cells co-infected with IAV and HKSP. Additionally, IFN-β expression also cannot be the reason for the inhibition as the mRNA levels were not above basal levels. Indeed, the type-I IFN amplification was so low, that the x-axis had to be split into sections for the amplification to be observed against the Poly(I:C) control.

As weak IFN-α and IFN-β gene expression was detected through qPCR, ELISA was used to detect for the presence of IFN-α and IFN-β protein production. Supernatants from treated samples were analysed to determine the levels of secreted IFN-α and IFN-β cytokines. Very low levels of both IFN-α and IFN-β protein was detected, apart from

Poly(I:C) control samples, which induced robust levels of IFN-α and IFN-β. Low levels of IFN-α protein were secreted by cells co-treated with H1N1 and HKSP, but no IFN-α protein was detected in cells co-treated with H3N2 and HKSP. Levels of IFN-β protein was detected in cells co-treated with HKSP and IAV, however these were below levels in untreated cells. The presence of low levels of IFN-α protein by cells co-treated with HKSP and H1N1 cannot be responsible for the inhibition of HKSP responses as little to no IFN-α protein was detected in cells co-treated with HKSP and H3N2, where the same inhibition occurs. The presence of IFN-β protein cannot explain the attenuation of the Th1 and Th17 responses as these are below basal levels, which were present in all cells, including untreated and HKSP treated cells, in which we observed robust Th17 innate immune responses. In addition, a previous study in human cells demonstrated that basal levels of IFN-β are insufficient to inhibit Th17 responses [412]. The low levels of type I IFN being produced may be due to the viral component NS1, which is synthesised during infection and is known to inhibit the production of type I IFN [38,54]. These results suggest that type I IFN production is not responsible for the early inhibition of *S.p.* by IAV.

As the inhibition of HKSP responses does not appear to be due to type I IFN, it points to IAV having a direct effect on immune responses. To investigate possible pathways which IAV could be targeting, we researched how the immune response to *S.p*. is usually mounted. Toll-Like Receptors are extremely important in the recognition of both IAV and *S.p.* amongst many other pathogens. These TLRs trigger signalling cascades in APCs which determine the kind of adaptive immune response that will be elicited [116,137,138]. In this study, we have sought to determine if IAV specifically targets specific *S.p.* associated TLRs. In addition, work in mouse models have established that a TLR5 agonist can have beneficial therapeutic effects when combined with antibiotic administration in the treatment of IAV and *S.p.* co-infections [388]. Therefore, we have endeavoured to determine whether a TLR5 agonist can be used to restore IAV inhibited immune responses to *S.p.* in primary human monocytes [350]. It has been established that the TLRs involved in sensing *S.p.* are TLR2, TLR4, and TLR9 [96]. Therefore, we examined the effects of IAV on TLR induction of cytokines involved in the Th17 response to *S.p.* in human APCs. The results obtained demonstrate that IAV targets aspects of each of the *S.p.* associated TLR pathways.

The TLR2 and TLR9 pathways appear to be inhibited more strongly by IAV than the TLR4 pathway. However, the TLR4 mono-agonist was not as strong of an inducer of these cytokines. TLR2- and TLR9-induced IL-23 is inhibited by both strains of IAV. The inhibition of IL-23 is interesting as we have previously shown that IAV inhibits HKSP-induced IL-23 (Chapter 2). IL-23 is necessary for the expansion and commitment to the

Th17 lineage. IL-23 is particularly important in induction of the cytokine IL-17A, which is pivotal in the response to *S.p*. [413,414].

Both H1N1 and H3N2 inhibited TGF-β induction by each of the TLRs studied. TGF-β is a multifunctional cytokine which exhibits both pro-inflammatory and anti-inflammatory properties [177,415]. This cytokine is involved in the differentiation of both Th17 cells and T_{reg} cells in a concentration-dependent manner [416]. At low concentrations, TGF-β synergises with IL-6 to promote Th17 expression and at high concentrations, TGF-β represses Th17 expression in favour of T_{reg} expression [416]. As TLR-induced TGF-β has been inhibited by both strains of IAV, it may have a negative effect on Th17 differentiation. It is interesting that TLR agonism of TGF-β was inhibited by IAV, and yet in a previous study (and in Chapter 2), whole HKSP-induction of TGF-β was not inhibited by IAV [350]. It is possible that whole HKSP contains components that activate pathways related to TGF-β which IAV either cannot or does not target. As different TLRs often dimerise with each other [417], it may be that IAV can target individual (mono)TLR activation of TGF-β, but that it cannot exert the same inhibitory effects on TLRs when in heterodimer conformation (which is likely with whole *S.p.*). It should be noted that IL-6, the cytokine with which TGF-β synergises has not been affected in any way as it has neither been strongly induced by any of the TLR agonists or inhibited by IAV. It may be that IL-6 is not induced by mono-agonists. It may require heterodimer activation in order to be induced above basal level. TLR-induced IL-6, IL-1β, and IL-27 were not affected by IAV, thus suggesting that IAV is having a specific effect on TLR-induced IL-23 and TGF-β.

As IAV inhibited *S.p.*, TLR2, and TLR9 induction of IL-23, this cytokine was researched further to determine why IL-23 specifically may be targeted by IAV. IL-23 signals through IL-23R and IL-12Rβ1, which leads to phosphorylation of STAT3 [187]. Phosphorylation of STAT3 leads to induction of RORC, which is a Th17-specific transcription factor. RORC in turn, induces further expression of IL-23R, which increases IL-23 cytokine induction in a positive-feedback loop [187,190–193]. As RORC has been shown to be important in the induction of IL-23, we sought to determine if RORC induction by both TLR2 and TLR9 was inhibited by IAV. We have demonstrated that TLR2 agonist induction of RORC was not significantly inhibited by IAV. Indeed, TLR2 agonist induction of RORC was inconsistent, with one donor inducing very high levels of RORC in response to TLR2 agonism, whereas two donors did not induce RORC past basal levels. RORC has been shown to be induced at different time points, therefore, it is possible that induction of RORC in response to TLR2 agonism is peaking at an earlier time [206]. It may be of interest to explore different time-points with regard to TLR2 agonist induction

of RORC to give more clarity. Additionally, we have demonstrated that TLR9 agonist induction of RORC is significantly inhibited by IAV. The inhibition of RORC is most likely why TLR9-induction of IL-23 is inhibited, and may also be why *S.p.* induction of IL-23 is inhibited. As RORC expression is induced by phosphorylation of STAT3, and inhibited by phosphorylation of STAT1, the effect of IAV on STAT3 and STAT1 phosphorylation may shed further light on the mechanisms behind IAV inhibition of *S.p.* and associated TLR responses. IAV-mediated inhibition of TLR-induction of RORC has not been demonstrated by human APCs previously.

TLR5 agonism has been shown to elicit protection in mice against multiple bacterial pathogens including *Clostridium difficile*, vancomycin-resistant *Enterococcus*, and *S.p.* [385–387]. Additonally, TLR5 agonism has improved the efficacy of antibiotics in treating IAV and *S.p.* co-infections in mice [388]. TLR5 is activated in response to flagellin [418], and therefore is not activated in response to *S.p.* infections. Due to the encouraging results using TLR5 agonism as a treatment in mice, we sought to investigate what effect TLR5 agonism may have on immune responses *S.p*. and IAV co-infection in human APCs.

We have established that the TLR5 pathway is not targeted by IAV as cytokine secretion is not impaired by IAV infection. Indeed, IL-23, which is inhibited by IAV, is induced robustly by a TLR5 agonist, even in the presence of IAV. As this pathway is not affected by IAV and to address the gap in the research using human models, we examined TLR5 agonism in a simulated co-infection with IAV and *S.p.* in human APCs. TLR5 agonism restored IAV-inhibited levels of HKSP-induced IL-23 and IL-27, and induced levels of IL-12p70 to above those observed in HKSP- treated cells alone.

In this chapter, we have demonstrated that IAV infection can directly inhibit immune responses, which is independent of anti-viral type I IFNs. Here, we are presenting a novel mechanism in restoring immune responses to *S.p.* in human APCs. The effect of IAV infection on *S.p.* associated TLRs has not been examined in human APCs previously. Additionally, we have also presented a possible mechanism where IAV is targeting the normal immune response to *S.p.* through the RORC analysis. This may be beneficial in determining new therapeutic targets.

Results in mouse models have been very promising, however due to differences in fundamental immune responses between mice and humans, it is imperative that similar investigations be carried out in human models. The results obtained in our human *ex vivo* model corroborate those observed in mouse models, and additionally have provided some mechanistic insight as to how IAV may be inhibiting immune responses to *S.p*. This study highlights the importance of the additional treatment strategies that may be possible by utilising certain TLR agonists. However, caution must be used when

considering the therapeutic benefits of this agonist, as increases in the cytokine IL-23 and other Th17 polarising cytokines have been associated with pathogenesis. This includes the development of inflammation in a range of autoimmune diseases such as rheumatoid arthritis, psoriasis, and Crohn's disease [198,295–297].

4.0 THE EFFECT OF HAEMAGGLUTININ TREATMENT ON RESPONSES TO *STREPTOCOCCUS PNEUMONIAE* AND TLR AGONISM IN HUMAN CELLS

4.1 INTRODUCTION

Previous studies carried out in the Viral Immunology Lab demonstrated that the IAV glycoprotein, HA, downregulated bacterial LPS-induced IL-12p70 in mouse bone-marrow derived DCs (BMDCs) [341]. This study aimed to explore the role of immunomodulation in the enhanced predisposition to bacterial superinfection during IAV infection [341]. HA is a key viral antigen, which along with NA is a glycoprotein on the surface of IAV [33]. HA plays a pivotal role in mediating viral entry into host cells, whilst NA removes host sialic acid residues and helps to release newly formed virions from infected cells [33]. Additionally, HA is commonly used as a target in the development of vaccines. Vaccines are designed to target both structural elements of the HA protein: the head and the stalk. The head is the main target of antibodies which protect against influenza [6,419]. Vaccination is the principal tactic in the prevention and regulation of influenza infections, but due to the constant mutating virus, vaccines must be reformulated annually [6,7].

In Chapter 2, we presented results which demonstrate that in human APCs and T cells, IAV inhibits *S.p.* induced innate and adaptive cytokines. Furthermore, this inhibition was not due to elevated levels of anti-inflammatory cytokines or an increase in apoptosis. In Chapter 3, we subsequently established that this inhibition was also not due to inhibitory effects of type I IFNs. In addition, we determined that the inhibition may be due to downward pressure on *S.p.* associated TLRs by IAV. As previous work using HA was carried out using a gram-negative bacteria in a murine model [341], the effect of HA on responses to *S.p.*, which is gram-positive was examined in our human *ex vivo* model to determine if HA may be key in the IAV-inhibition of *S.p.* responses. As the influenza vaccine and pneumococcal vaccines are frequently administered simultaneously [108], the effect of HA on pneumococcal responses was of particular interest. Importantly, as IAV has been shown to target specific TLR pathways (as described in Chapter 3), the effect of HA on the same TLR agonists was examined. Furthermore, as a TLR5 agonist (flagellin) is currently being developed as an adjuvant to influenza vaccines [420–422], the effect of HA on TLR5 agonism was also determined.

This research may clarify whether different HA conformations influence how immunosuppressive certain strains of IAV are. This may be beneficial in the prediction

of how different strains may be more or less likely to predispose individuals to secondary bacterial infections.

Specific aims of this chapter:

- Establish innate and adaptive immune responses to new batches of influenza HA,
- Compare immune responses to H1N1 HA with H3N2 HA,
- Further investigate the effect of new HA batches on responses to *S.p.*,
- Determine the effect of HA treatment on *S.p.* associated TLR agonism, and
- Study the effect of TLR5 agonism on cells treated with HA.

4.2 MATERIALS AND METHODS

Table 4.1 List of additional reagents to Table 2.1 (Chapter 2) and Table 3.1 (Chapter 3) used in this study

Product	Catalogue Number	Company
H1HN1 A/PR/8/34 HA	N/A	NIBSC, UK
H3N2 A/Uruguay/716/2007 HA	N/A	

4.2.1 ISOLATION OF PRIMARY HUMAN IMMUNE CELLS

Human PBMCs were isolated from healthy human donors as per Section 2.2.1. and cell yield was determined as per Section 2.2.2.

4.2.2 SEPARATION OF CD14$^+$ AND CD3$^+$ CELLS FROM PBMCs USING MICROBEAD SEPARATION

CD14$^+$ APCs and CD3$^+$ T cells were purified from PBMCs as per Section 2.2.3.

4.2.3 STREPTOCOCCUS PNEUMONIAE STIMULATION OF CD14$^+$ ANTIGEN PRESENTING CELLS

CD14$^+$ APCs were exposed to HKSP as per Section 2.2.5.

4.2.4 HA TREATMENT OF CD14$^+$ ANTIGEN PRESENTING CELLS

CD14$^+$ APCs which had been separated from PBMCs (as per Section 2.2.3) were cultured in cRPMI at a concentration of 1x10^6 cells/ml. Two strains of HA were used in this study: H1N1 HA (A/Puerto-Rico/8/34) and H3N2 (A/Uruguay/716/2007) provided by Ruth Harvey (NIBSC, UK). CD14$^+$ APCs at a density of 1x10^6 cells/ml were treated with H1N1 HA or H3N2 HA at concentrations of 1 μg/ml and 3 μg/ml, alone or in combination with HKSP for 24 hours (as per Section 2.2.5).

4.2.5 TOLL LIKE RECEPTOR AGONIST STIMULATIONS

TLR treatments were performed as per Section 3.2.6.

4.2.6 CO-CULTURE ASSAY

Co-cultures of $CD3^+$ T cells and $CD14^+$ APCs were performed as per Section 2.2.6.

4.2.7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Supernatant from treated cells was used to detect for cytokines using ELISA as per Section 2.2.7.

4.2.8 STATISTICAL ANALYSIS

Statistical analyses were performed as per Section 2.2.11.

4.3 RESULTS

4.3.1 HAEMAGGLUTININ TREATMENT INHIBITS STREPTOCOCCUS PNEUMONIAE INDUCED INNATE POLARISING CYTOKINES

A key component of IAV is the surface glycoprotein HA, of which there are 18 distinct subtypes [33,35,36]. Changes in HA subtypes are part of what causes the emergence of new strains of IAV, which result in not only new seasonal IAV strains but also pandemic strains [83,84]. As previous studies have demonstrated that HA treatment inhibited Gram-negative LPS-induction of IL-12p35 in mouse BMDCs [341], we sought to investigate if HA treatment may also inhibit Gram-positive *S.p.* induction of cytokines in our human *ex vivo* model.

Here, it is shown that HA treatment inhibits certain HKSP induced innate polarising cytokines in primary human immune cells. The inhibition has occurred in cytokines involved in the Th17 response and Th1 response. Supernatants from treated $CD14^+$ APCs were used to detect for cytokines using ELISA (as per Section 4.2.7). HA treatment inhibited HKSP-induction of IL-27 (Figure 4.1 A) and IL-12p70 (Figure 4.1 B) (n=5). HA treatment did not inhibit HKSP-induction of IL-23 (Figure 4.2 A), IL-6 (Figure 4.2 B), or IL-1β (Figure 4.2 C) (n=5). Results generated using new batches of HA were compared to those generated using previous batches of HA to determine if similar innate responses between different batches and subtypes occurred. The results show that similar immune responses occur with the new HA batch when compared to the previous HA batches (Appendix 2.1).

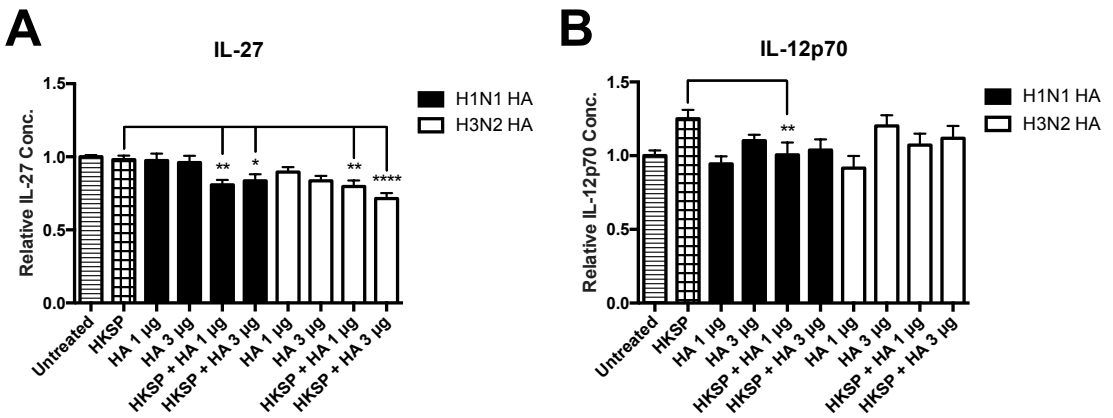

Figure 4.1 Influenza HA attenuates HKSP induction of IL-27 and IL-12p70. The levels of (A) IL-27 and (B) IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, H1N1 HA (1 µg/ml or 3 µg/ml) or H3N2 HA (1 µg/ml or 3 µg/ml) alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either H1N1 HA (1 µg/ml or 3 µg/ml) or H3N2 HA (1 µg/ml or 3 µg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*$p<0.05$, **$p<0.01$, ****$p<0.0001$).

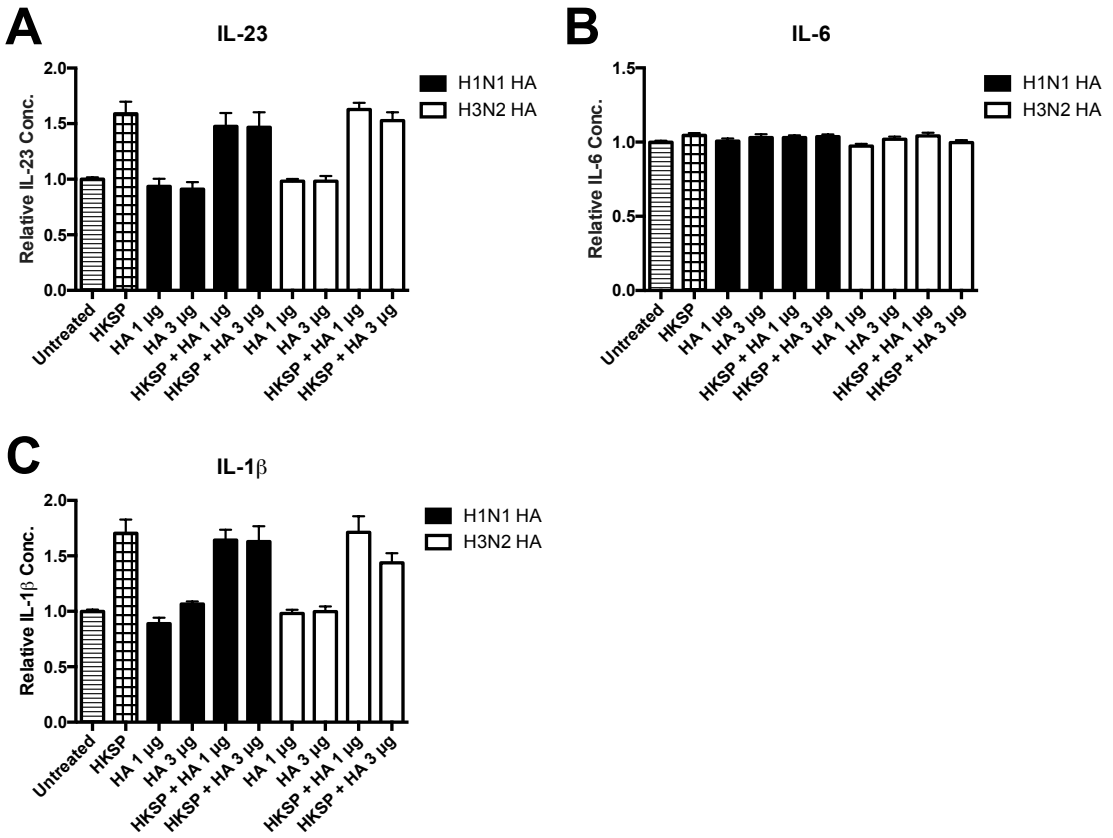

Figure 4.2 Influenza HA does not affect HKSP induction of IL-23, IL-6, and IL-1β. The levels of (A) IL-23, (B) IL-6, and (C) IL-1β secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

4.3.2 INHIBITION BY HAEMAGGLUTININ TREATMENT IS NOT DUE TO ANTI-INFLAMMATORY EFFECTS OF IL-10 AND TGF-β

To assess if HA treatment altered levels of HKSP-induced anti-inflammatory cytokines, the levels of IL-10 and TGF-β were assessed. As mentioned previously, IL-10 can induce T_{reg} cells which limit inflammatory responses [211,243,244]. TGF-β can exert both pro- and anti-inflammatory effects [176,208]. The 3 μg dose of H3N2 HA significantly inhibited HKSP-induced IL-10 (Figure 4.3 A) (n=5). HKSP-induction of TGF-β was not affected by HA treatment (Figure 4.3 B) (n=5). The inhibition of the Th17 response cannot be

attributed to the anti-inflammatory effects of IL-10 and TGF-β as HKSP induction of IL-10 was also inhibited and no increase in TGF-β was observed.

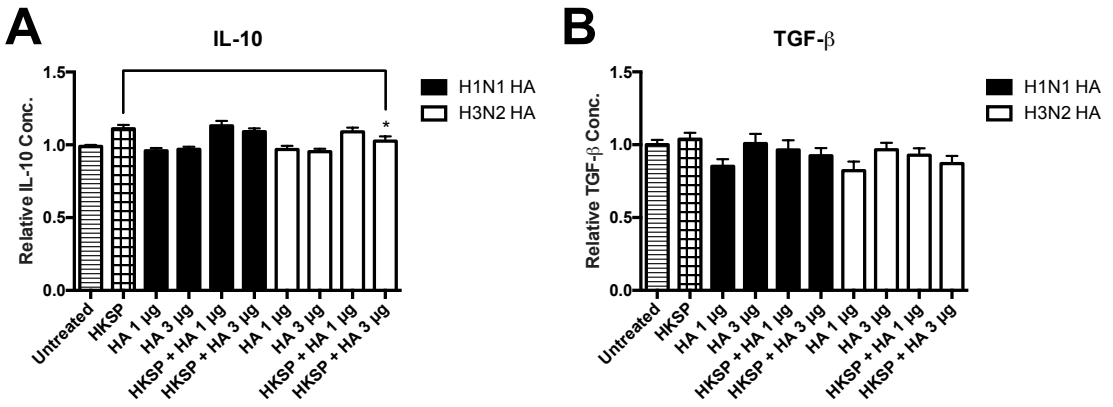

Figure 4.3 Influenza HA does not increase levels of IL-10 and TGF-β. The levels of (A) IL-10 and (B) TGF-β secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, H1N1 HA (1 µg/ml or 3 µg/ml) or H3N2 HA (1 µg/ml or 3 µg/ml) alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 5 donors (n=5). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either H1N1 HA (1 µg/ml or 3 µg/ml) or H3N2 HA (1 µg/ml or 3 µg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*$p<0.05$).

4.3.3 HAEMAGGLUTININ TREATMENT INHIBITS ADAPTIVE TH17 AND TH1 RESPONSES TO STREPTOCOCCUS PNEUMONIAE

As mentioned previously, HA is a key component of IAV [33,35,36]. Here, it is shown that HA treatment inhibits adaptive immune responses to HKSP in a co-culture of $CD14^+$ APCs and $CD3^+$ T cells. The inhibition has occurred in cytokines involved in the Th17 and Th1 responses. Supernatants from treated co-cultures (APC-T cells) were used to detect for cytokines using ELISA (as per Section 4.2.7). HA treatment inhibited HKSP-induction of IL-17A (Figure 4.4 A) and IFN-γ (Figure 4.4 B) (n=3). This inhibition occurred in the absence of elevated anti-inflammatory cytokines, TGF-β (Figure 4.5 A) and IL-10 (Figure 4.5 B) (n=3). Adaptive immune responses to new batches of HA were compared to adaptive immune responses to previous batches of HA. Results indicate that very similar immune responses occur to both previous batches and new batches of HA (Appendix 2.2).

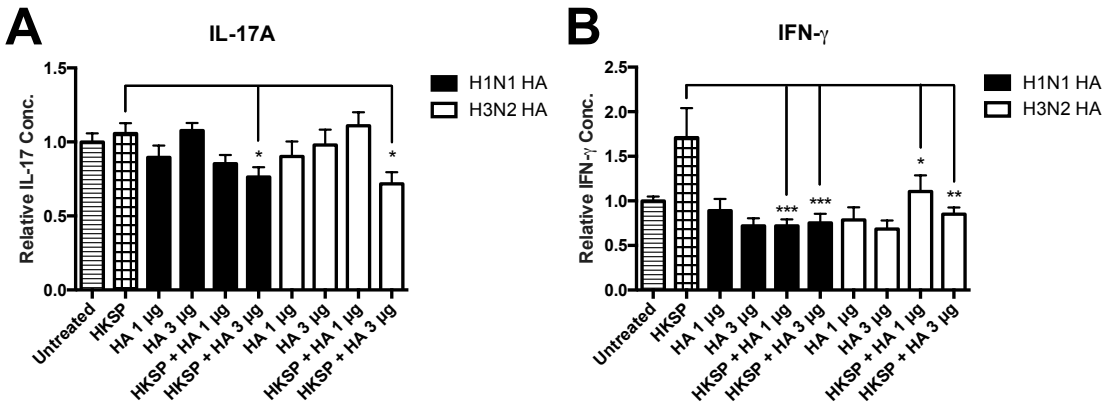

Figure 4.4 Influenza HA attenuates HKSP induction of IL-17A and IFN-γ. The levels of (A) IL-17A and (B) IFN-γ secreted by $CD14^+$ APC and $CD3^+$ T cell co-culture following 24 hr treatment with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*p<0.05, **<p0.01, ***p<0.001).

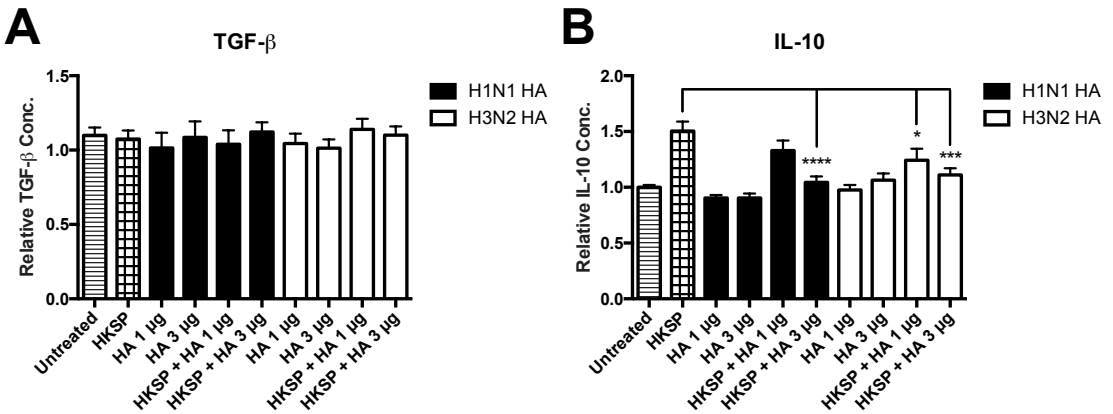

Figure 4.5 Influenza HA does not increase levels of HKSP induction of TGF-β and IL-10. The levels of (A) TGF-β and (B) IL-10 secreted by $CD14^+$ APC and $CD3^+$ T cell co-culture following 24 hr treatment with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with HKSP or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to HKSP alone versus cells exposed to HKSP in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*p<0.05, ***p<0.001. ****p<0.0001).

4.3.4 HAEMAGGLUTININ TREATMENT INHIBITS INNATE TLR2 AGONIST INDUCTION OF TGF-β

As demonstrated in Chapter 3, IAV infection inhibited TLR2-induced innate polarising cytokines. As HA treatment has inhibited HKSP-induction of both IL-27 and IL-12p70, the effect of HA on TLR2 agonism was examined. Here, it is shown that HA treatment (both subtypes) at a concentration of 3 μg/ml inhibits TLR2-induction of TGF-β (Figure 4.6) (n=3), however other cytokines tested were not affected (Figure 4.7) (n=3). This inhibition was not due to elevated levels of IL-10 (Figure 4.8) (n=3).

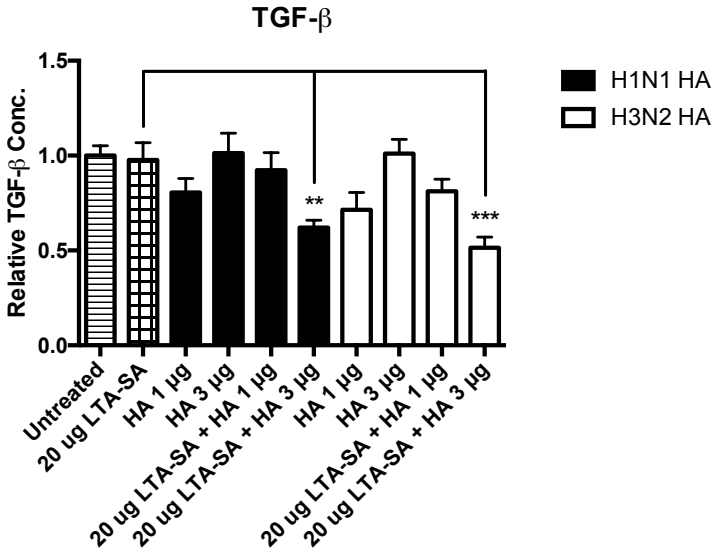

Figure 4.6 Influenza HA attenuates LTA-SA induction of TGF-β. The levels of TGF-β secreted by $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with LTA-SA or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to LTA-SA alone versus cells exposed to LTA-SA in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (**$p<0.01$, ***$p<0.001$).

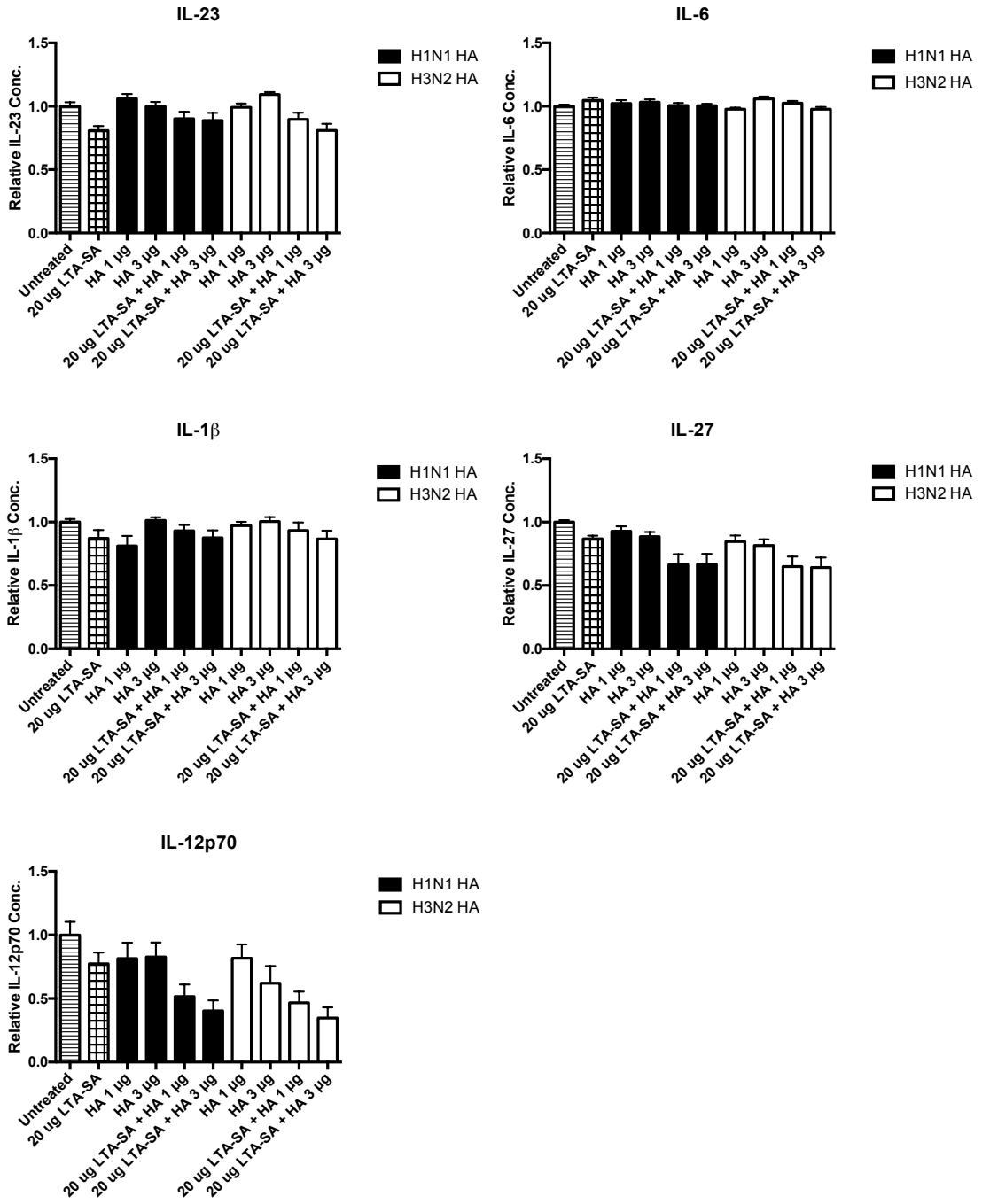

Figure 4.7 Influenza HA does not attenuate LTA-SA induction of cytokines. The levels of IL-23, IL-6, IL-1β, IL-27, and IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with LTA-SA or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to LTA-SA alone versus cells exposed to LTA-SA in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

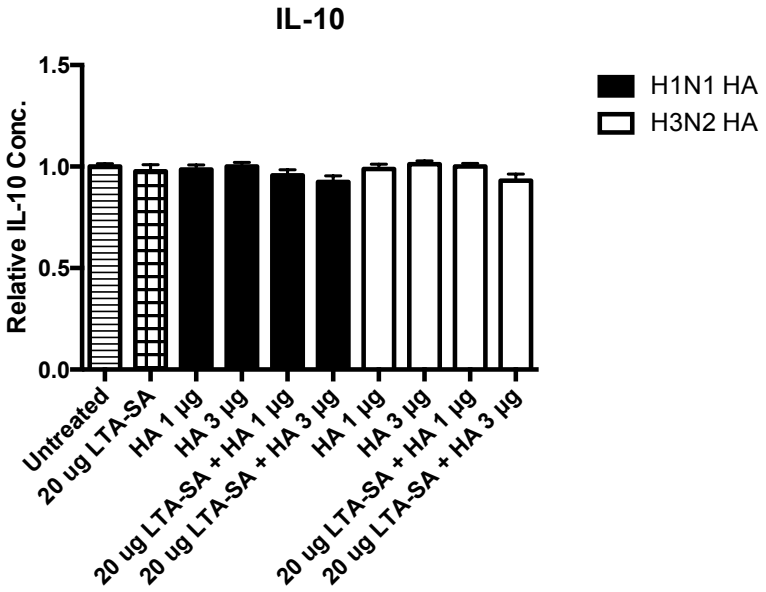

Figure 4.8 Influenza HA does not cause elevate levels of LTA-SA induction of IL-10. The levels of IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR2 agonist (LTA-SA), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with LTA-SA or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels by cells exposed to LTA-SA alone versus cells exposed to LTA-SA in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

4.3.5 HAEMAGGLUTININ TREATMENT DOES NOT INHIBIT INNATE TLR4 AGONISM

In Chapter 3, it was shown that IAV infection inhibited TLR4-induced innate TGF-β. To determine whether HA might be playing a role in this inhibition, the effect of HA treatment on TLR4 agonism was examined. Here, it is shown that HA treatment (both subtypes) did not affect (either negatively or positively) TLR4-induction of cytokines (Figure 4.9) (n=3).

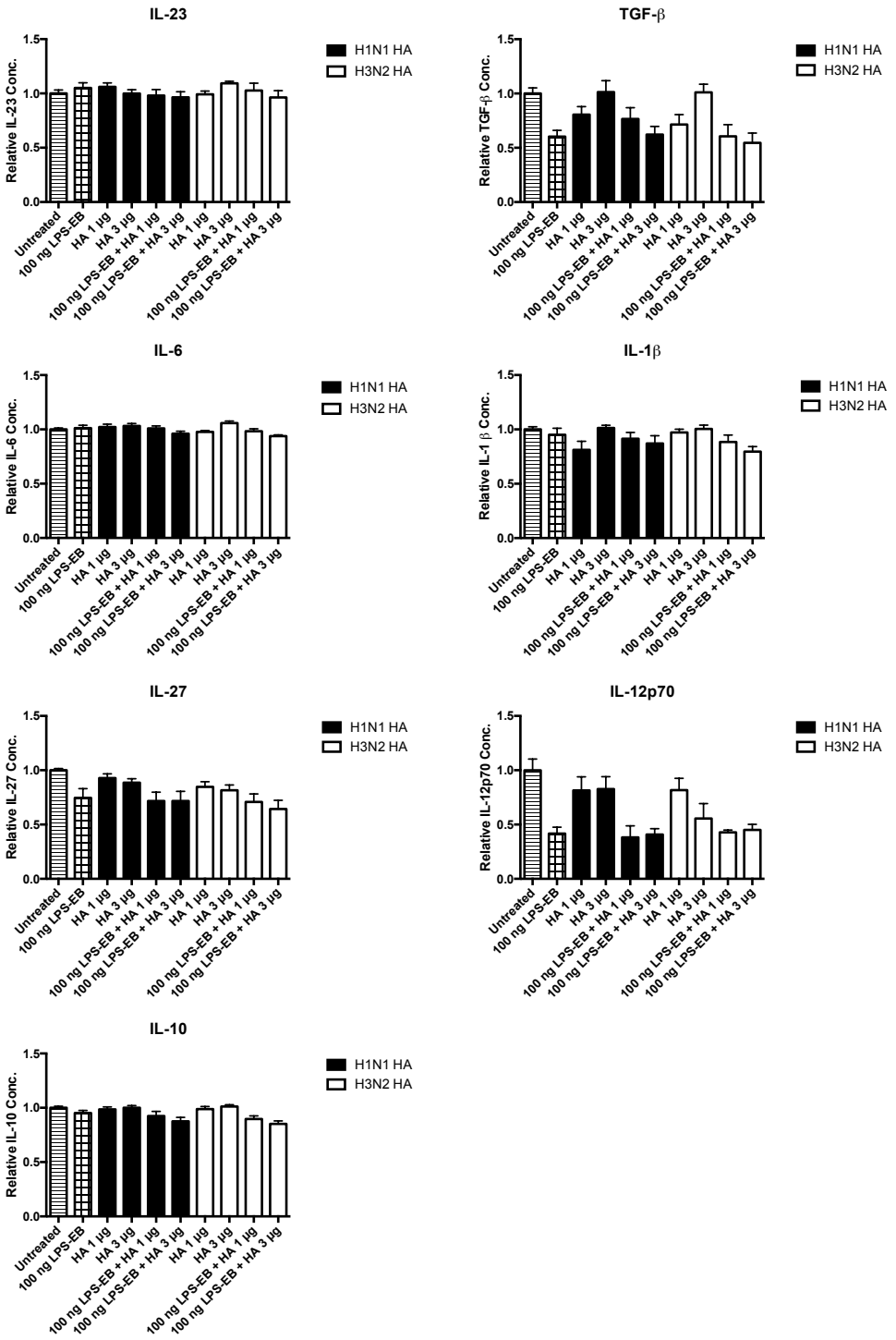

Figure 4.9 Influenza HA does not attenuate LTA-SA induction of cytokines. The levels of IL-23, TGF-β IL-6, IL-1β, IL-27, IL-12p70, and IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR4 agonist (LPS-EB), H1N1 HA (1 µg/ml or 3 µg/ml) or H3N2 HA (1 µg/ml or 3 µg/ml) alone or in combination with LPS-EB or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to LPS-EB alone versus cells exposed to LPS-EB in combination with either H1N1 HA (1 µg/ml or 3 µg/ml) or H3N2 HA (1 µg/ml or 3 µg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

4.3.6 HAEMAGGLUTININ TREATMENT INHIBITS INNATE TLR9 AGONIST INDUCTION OF IL-23

In Chapter 3, live IAV inhibited TLR9-induction of both IL-23 and TGF-β. To further establish if HA was a factor in this inhibition, cells were treated with a TLR9 agonist and different concentrations and subtypes of HA. It was established that H1N1 HA treatment at both concentrations inhibited TLR9-induction of IL-23 (Figure 4.10) (n=3). HA treatment had no effect on TLR9-induction of other cytokines (Figure 4.11) (n=3). No increase in anti-inflammatory IL-10 was observed in any treatments (Figure 4.12) (n=3).

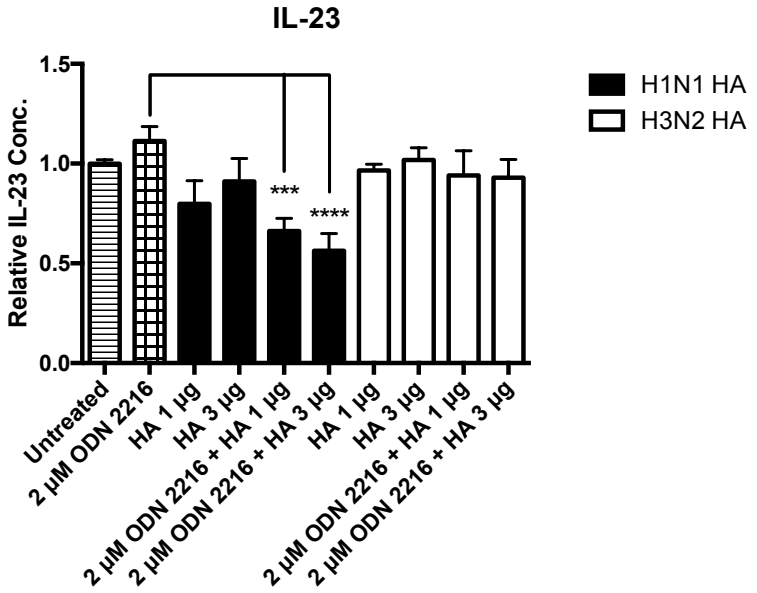

Figure 4.10 Influenza HA attenuates ODN 2216 induction of IL-23. The levels of IL-23 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR9 agonist (ODN 2216), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with ODN 2216 or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to ODN 2216 alone versus cells exposed to ODN 2216 in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (***$p<0.001$, ****$p<0.0001$).

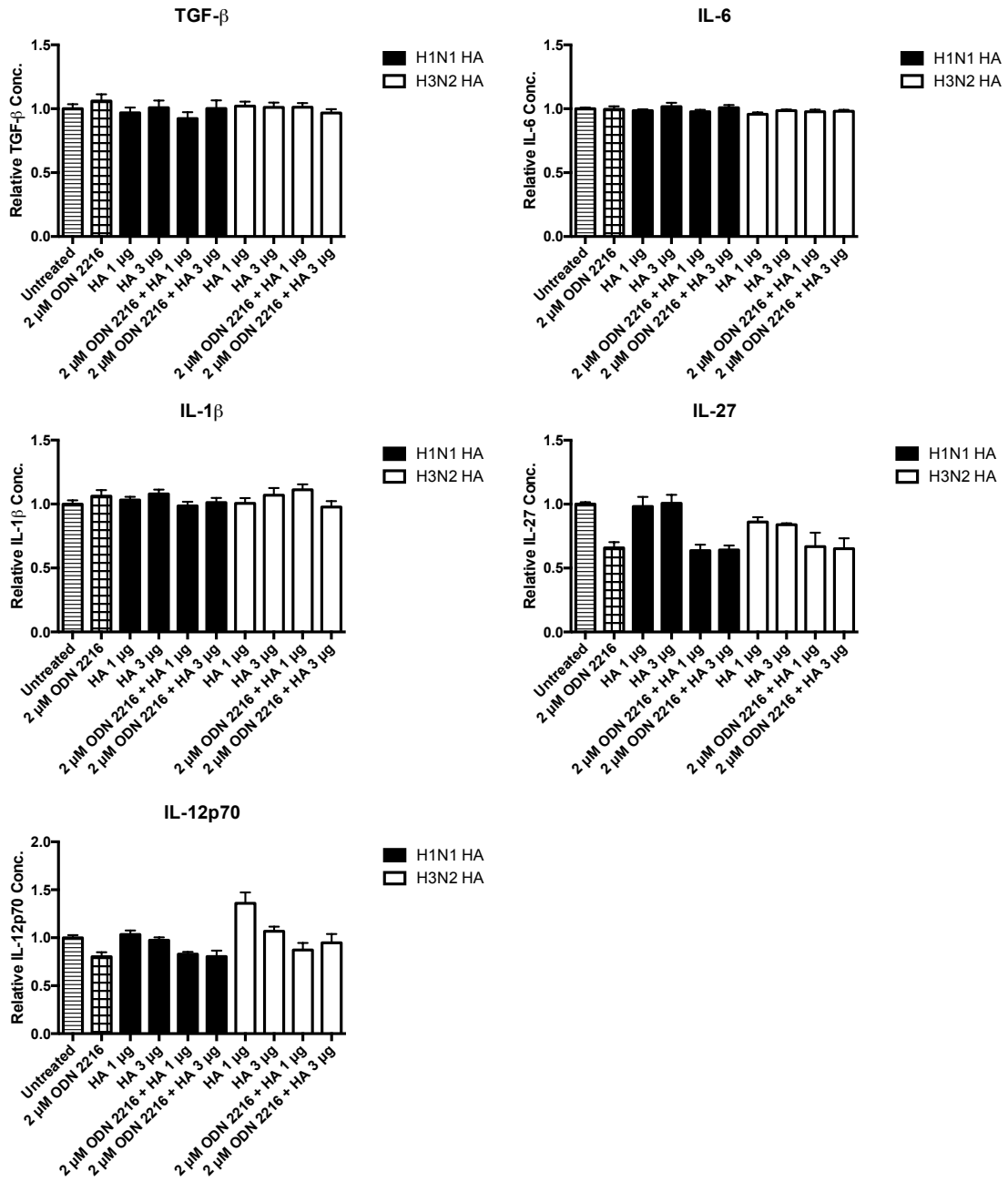

Figure 4.11 Influenza HA does not attenuate ODN 2216 induction of cytokines. The levels of TGF-β IL-6, IL-1β, IL-27, and IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR9 agonist (ODN 2216), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with ODN 2216 or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels by cells exposed to ODN 2216 alone versus cells exposed to ODN 2216 in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

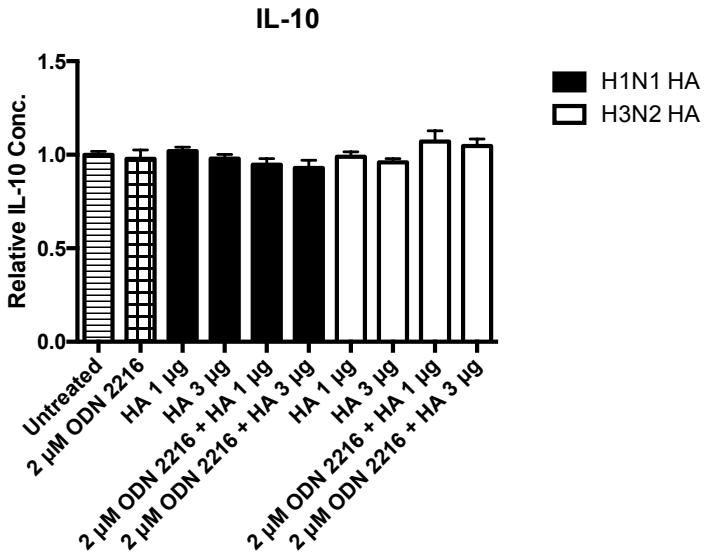

Figure 4.12 Influenza HA does not elevate levels of ODN 2216 induced of IL-10. The levels of IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR9 agonist (ODN 2216), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with ODN 2216 or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels by cells exposed to ODN 2216 alone versus cells exposed to ODN 2216 in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

4.3.7 HAEMAGGLUTININ TREATMENT DOES NOT INHIBIT INNATE TLR5 AGONISM

A TLR5 agonist as an adjuvant to the influenza vaccine is currently being developed and clinical trials are ongoing [420–422]. In Chapter 3, live IAV was shown to not have an inhibitory effect on TLR5 agonism. However, as HA is a common component of IAV vaccines, and TLR5 agonism has also been proposed for use prophylactically and in vaccines, any possible interactions between HA and flagellin should be explored [385]. It was established that HA treatment did not inhibit TLR5-induction of Th17 and Th1 polarising cytokines (Figure 4.13) (n=3). Additionally, no increase in anti-inflammatory IL-10 was observed across any treatments (Figure 4.13) (n=3).

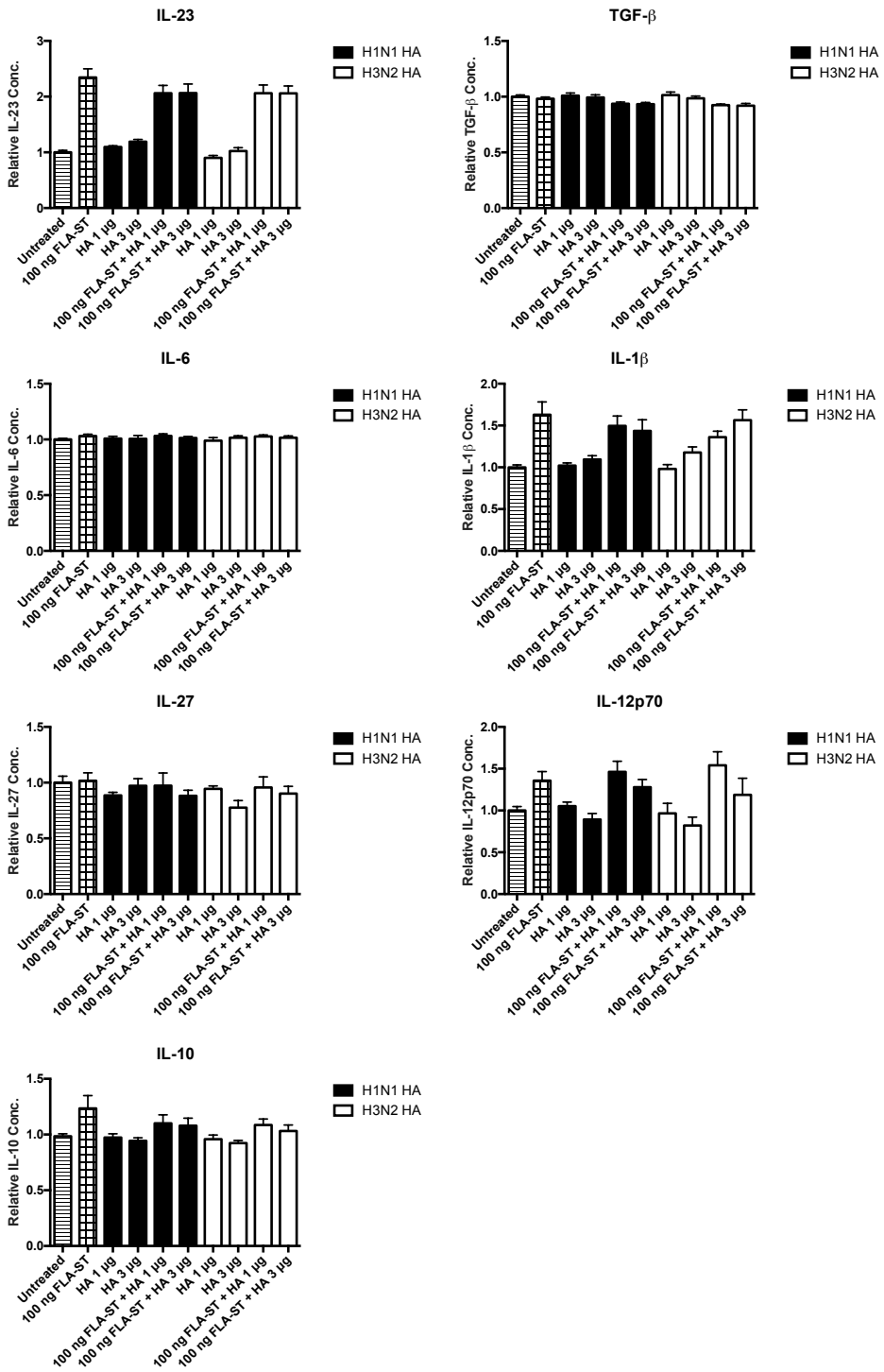

Figure 4.13 Influenza HA does not attenuate FLA-ST induction of cytokines. The levels of IL-23, TGF-β IL-6, IL-1β, IL-27, IL-12p70, and IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with a TLR5 agonist (FLA-ST), H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) alone or in combination with FLA-ST or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells exposed to FLA-ST alone versus cells exposed to FLA-ST in combination with either H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

4.3.8 TREATMENT WITH A TLR5 AGONIST RESTORES INHIBITED IMMUNE RESPONSES TO HKSP DURING HAEMAGGLUTININ TREATMENT IN HUMAN ANTIGEN PRESENTING CELLS

Pneumococcal vaccines are often administered simultaneously with influenza vaccines, however as new vaccines may soon, not only contain HA, but also flagellin as an adjuvant, the immune responses to this combination was explored. Additionally, as demonstrated earlier in this chapter, HA treatment inhibited HKSP-induction of IL-27 and IL-12p70. In Chapter 3, TLR5 agonism has been shown to restore IAV-inhibited immune responses to HKSP, therefore the effect of TLR5 agonism on HA and HKSP co-treatment was examined. Here, it is shown that TLR5 agonism restored HKSP-induced IL-27 (Figure 4.14 A) and IL-12p70 (Figure 4.14 B) (n=3). Additionally, TLR5 agonism increased levels of IL-23 (Figure 4.15 A) and IL-1β (Figure 4.15 B) to above those observed in HKSP alone-treated cells. No significant effects occurred with respect to secretion of IL-6, TGF-β and IL-10 (Figure 4.16).

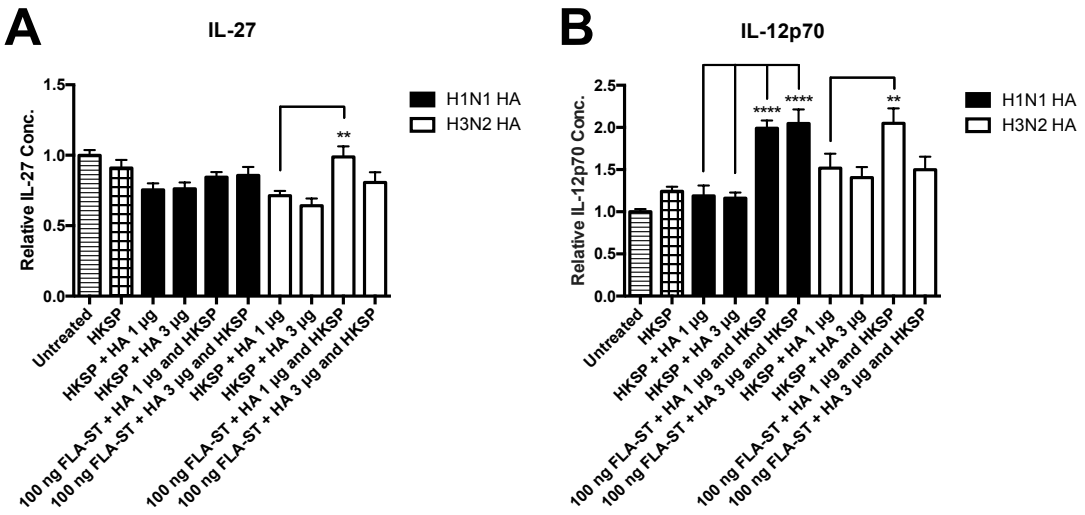

Figure 4.14 TLR5 agonism restores inhibited HKSP-induction of IL-27 and IL-12p70 during HA treatment. The levels of (A) IL-27 and (B) IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP and a TLR5 agonist (FLA-ST), or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP in combination with H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) versus cells treated with H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP and FLA-ST by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (**p<0.01, ****p<0.0001).

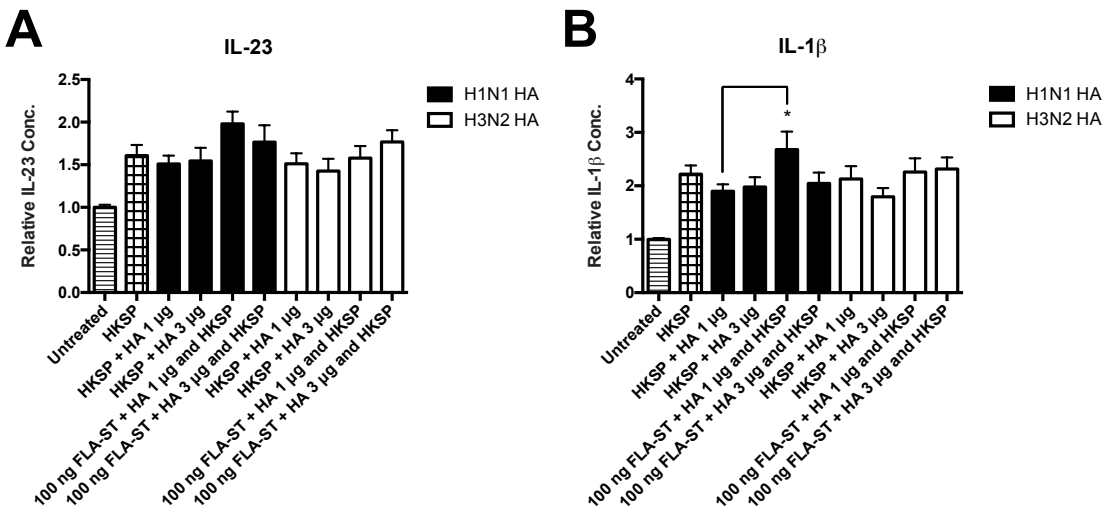

Figure 4.15 TLR5 increases induction of IL-23 and IL-1β during HKSP and HA co-treatment. The levels of (A) IL-23 and (B) IL-1β secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP and a TLR5 agonist (FLA-ST), or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 experimental repeats of each treatment in the same donor (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP in combination with H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) versus cells treated with H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP and FLA-ST by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing (*$p<0.05$).

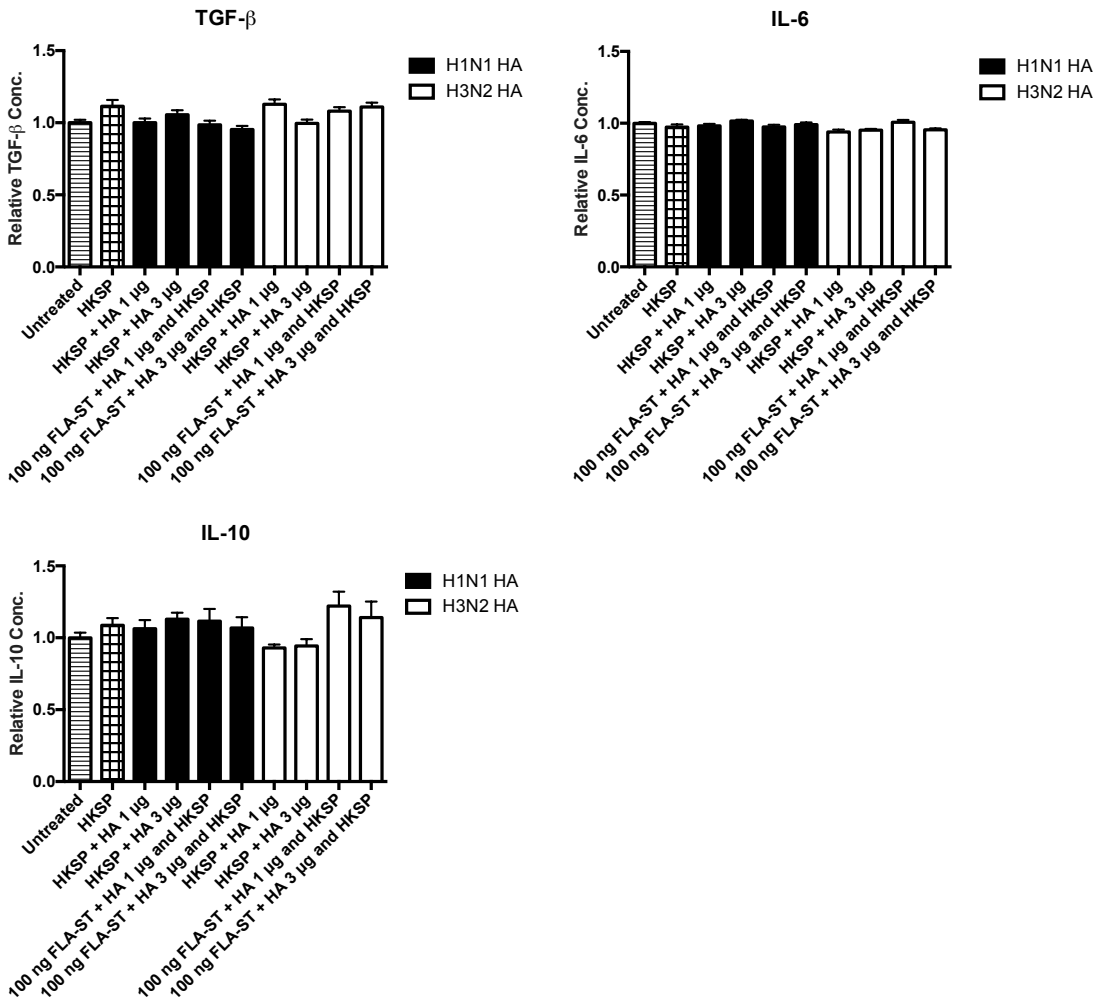

Figure 4.16 TLR5 agonism does not affect induction of cytokines during HKSP and HA co-treatment. The levels of TGF-β IL-6, and IL-10 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP, H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP and a TLR5 agonist (FLA-ST), or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 technical repeats of each treatment in every donor. Each ELISA represents normalised results from 3 donors (n=3). Statistical analyses were performed to compare cytokine levels secreted by cells treated with HKSP in combination with H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) versus cells treated with H1N1 HA (1 μg/ml or 3 μg/ml) or H3N2 HA (1 μg/ml or 3 μg/ml) in combination with HKSP and FLA-ST by fitting a One-Way ANOVA to the data and using a Sidak test to adjust for multiple testing.

4.4 DISCUSSION

An important component of IAV is the surface glycoprotein HA, which along with NA, are key viral antigens [33]. The HA protein serves as a target for vaccine development, which is the principal tactic in the prevention and regulation of influenza infections. However, vaccines must be reformulated annually due to the constant mutations within the virus [6,7]. As HA treatment has been shown to inhibit Gram-negative LPS-induction of IL-12p35 in BMDCs [341], the effect of HA treatment on Gram-positive *S.p.* induction of key polarising cytokines involved in the Th17 and Th1 responses were examined.

Supernatants from immune cells were analysed to determine the levels of secreted cytokines. Both H1N1 and H3N2 HA inhibited HKSP-induced IL-27, whereas only H1N1 HA inhibited HKSP-induced IL-12p70. HKSP-induction of IL-27 was inhibited by both subtypes of HA and at both concentrations. The 1 μg dose of H1N1 HA inhibited HKSP-induction of IL-12p70. HA treatment did not inhibit HKSP-induction of IL-23, IL-6, or IL-1β. IL-12p70 is a Th1 polarising cytokine and additionally IL-27 has been shown to have a role in Th1 immunity [354,423]. As HA has inhibited HKSP-induction of both IL-12p70 and IL-27, but not any other cytokines, it appears that HA targets cytokines involved in the Th1 response more than the Th17 response. It may be that the viral component is at least partially responsible for IAV inhibition of Th1 responses to *S.p.*, but it does not play a role in the inhibition of Th17 responses.

As IL-10 and TGF-β can exert anti-inflammatory effects, levels of these cytokines were analysed to determine a role, if any, in the inhibition of these innate cytokines. Elevated levels of IL-10 and TGF-β were not detected in treated samples in this study. In fact, HKSP-induction of IL-10 was slightly inhibited by the 3 μg dose of H3N2 HA (although fold-change was very low). Anti-inflammatory effects of TGF-β and IL-10 cannot be responsible for any inhibition of HKSP responses by HA treatment. This corroborates previous research which showed that pre-incubation of BMDCs with anti-IL-10 did not revert HA inhibition of LPS induction of IL-12 [341].

To examine adaptive immune responses to HA, both IL-17A and IFN-γ were studied. As mentioned before, these were chosen as they are the key cytokines in the Th17 and Th1 responses to bacteria [116,137–142]. The 3 μg concentration of H1N1 and H3N2 HA inhibited HKSP-induced IL-17A. All HA treatments inhibited HKSP-induced IFN-γ. As with live IAV, no differences in TGF-β levels were observed. HKSP-induction of IL-10 was not elevated in responses to HA treatment. Indeed, HKSP-induced IL-10 was inhibited by H1N1 and H3N2 HA treatment. As with studies performed on the innate response, the effect of HA treatment seems to be more immunosuppressive on the Th1

cytokine, IFN-γ than on the Th17 cytokine, IL-17A. Additionally, H1N1 HA seems to have a more immunosuppressive effect than H3N2 HA.

Although, HA can exert certain inhibitory effects, it seems unlikely that this viral protein is the main cause behind the strong inhibitory effects of live IAV on pneumococcus responses (as described in Chapter 2) as HA does not inhibit HKSP-induction of cytokines such as IL-23 and IL-6, which are key in Th17 differentiation. A different viral protein, NA, may also be playing a role and it would be of interest to perform similar experiments with NA treatment and compare them to results obtained using HA as above. Another viral component that may be key is NS1, which is known to inhibit immune responses such as the production of type I IFN [38,54]. As very low levels of type I IFN was detected in cells (described in Chapter 3), it may be that NS1 is exerting inhibitory effects. Therefore, it may be of interest to try and determine immune responses to NS1.

As in Chapter 3, the effect of IAV on TLR induction of cytokines was examined, we sought to establish if the observed inhibition may in part, be due to the IAV glycoprotein HA, which was already shown to inhibit HKSP-induction of IL-27 and IL-12p70, as detailed above. Supernatants of $CD14^+$ APCs treated with various TLR agonists and HA at different concentrations were analysed. It has been demonstrated that both subtypes of HA at the 3 μg/ml concentration inhibited TLR2-induction of TGF-β. It is curious that TLR2-induction of TGF-β was inhibited by HA, and yet no inhibition of HKSP-induction of TGF-β by HA occurred. However, this phenomenon was also noted in Chapter 2 and 3, using live IAV (where IAV did not inhibit HKSP-induction of TGF-β but did inhibit TLR2-induction of TGF-β). As mentioned before, it is possibly due to the mono-agonist nature of the TLR2 agonist, which may be more susceptible to inhibition than whole HKSP, which likely stimulates multiple TLRs simultaneously. HA treatment may be somewhat responsible for the inhibition of TLR2-induced TGF-β by IAV. However, HA treatment is not the reason behind the IAV mediated inhibition of TLR2-induced IL-23 as no inhibition of TLR2 induction of IL-23 by HA occurred. This is similar to the results obtained for HKSP stimulations, where live IAV inhibited HKSP-induction of IL-23, but HA did not. HA did not affect TLR2-induction of any other cytokines studied, which corroborates the live IAV studies.

HA treatment had no effect on TLR4-induction of cytokines, which is interesting as live IAV inhibited TLR4-induction of TGF-β. It was previously noted that of the TLRs studied, TLR4 seemed less vulnerable to inhibition as only TGF-β was inhibited by IAV (as described in Chapter 3). The downward pressure by IAV on the TLR4 pathway cannot

be attributed to HA in this case, but may be due to a different viral component.

H1N1 HA treatment at both concentrations inhibited TLR9-induction of IL-23. However, no inhibition of TLR9-induced IL-23 occurred in response to H3N2 HA treatment. As noted in Chapter 3, IAV had a profound inhibitory effect on TLR9-induction of IL-23, therefore, it is unsurprising that HA should also inhibit TLR9 responses. Yet, as only the H1N1 HA inhibited responses, coupled with the fact that no inhibition of HKSP-induced IL-23 by HA occurred, this is rather unusual. But, again as mentioned before, mono-agonists are more susceptible to inhibition than whole HKSP, which signals through multiple receptors. There was no effect on TLR9-induction of other cytokines by HA treatment. TLR9-induction of TGF-β was not inhibited by HA, yet was inhibited by live IAV, therefore, a different viral component of IAV may be exerting the inhibitory effects of IAV. No increase in anti-inflammatory IL-10 was observed across any treatments.

HA treatment had no effect on TLR5 induction of innate cytokines. This is in keeping with previous results performed using live IAV (Chapter 3). This is encouraging due to a TLR5 agonist being used as an adjuvant for influenza vaccines [420–422]. As demonstrated in Chapter 3, the TLR5 agonist has been shown to restore inhibition of HKSP by IAV, therefore the effect of TLR5 agonism on HKSP inhibition by HA was explored. As HKSP-induced IL-27 and IL-12p70 were inhibited by HA, these were of particular interest. Stimulation with a TLR5 agonist restored HKSP-induced IL-27 and IL-12p70. In addition, TLR5 agonism increased levels of IL-23 and IL-1β to above those observed in HKSP (alone) treated cells. As with studies performed using IAV, TLR5 agonism appears to be very effective in circumventing inhibition of HKSP induction by HA. This shows promise, but the effects of TLR5 agonism on other viral components should perhaps be considered to determine how this circumvention may be occurring. The work presented in this Chapter is entirely novel and no other research has been published examining the effect of IAV HA treatment on *S.p.* associated TLRs in human cells. Additionally, the interaction between TLR5 agonism and HA treatment had not been previously demonstrated.

5.0 GENERAL DISCUSSION

Respiratory infections are the leading cause of disease worldwide [424]. Acute respiratory disease is most commonly caused by viral pathogens [21]. Influenza is one of the most prevalent respiratory viral pathogens [17]. Influenza A Virus is highly contagious and poses substantial public health problems due to its strong association with morbidity and mortality [18]. It is estimated that seasonal influenza virus is the cause of approximately half a million deaths annually [30,31]. Most of these deaths are due to secondary bacterial pneumonia [18,85]. It is widely believed that these co-infections are a major contributor to increased morbidity and mortality associated with both seasonal and pandemic outbreaks of influenza [18]. Such secondary bacterial infections are typically caused by capsular, extracellular bacteria such as *S.p.* and *Staphylococcus aureus* [1–3]. Th1 cells were originally thought to be the crucial cell group in clearing such bacterial infections [266]. However, more recently Th17 cells have been identified as pivotal in the effective clearance of *S.p.* from the lung [88]. In addition, further studies demonstrated that IAV infection inhibits the Th17 response in mice [2,349]. As mentioned previously, most of the research carried out in this area is performed in animal models, particularly mice [102,316–319]. However, such research can often be contradictory [102,316]. To overcome some of the drawbacks encountered with conventional mouse models, humanised mouse models have been developed. Humanised mouse models are developed by engrafting human cells (often PBMCs) and tissues into immunodeficient mice, resulting in the recreation of a functional human immune system in mice. This also means that human influenza viruses can be used as opposed to mouse-adapted viruses. However, as with all models, there are limitations such as the high variability between mice during the development of the humanised mouse model, and differences in the mechanisms of viral pathogenesis between humans and mice [425–429]. Although, mouse models are routinely used, many studies have proposed that ferrets are the most suitable animal to model influenza and bacterial co-infections as they are susceptible to infection with human influenza viruses, whereas most mouse models require prior host adaptation of the human viruses [324,325]. There are other limitations to using ferrets, which limit the group sizes, resulting in problems regarding statistical analyses [327]. Common *in vitro* models have involved culturing DCs in the presence of IL-4 and GM-CSF, or IFN-α to generate IL-4 DCs and IFN-DCs, respectively [328–332,336,337]. However, IL-4 DCs are unlikely to portray the cytokine environment at the site of a viral infection [332,333], and additionally, IFN-DCs have additional properties which mark them as distinct from DCs *in vivo* [336,338,339]. Due to this, our lab group sought to develop a human *ex vivo* immune model to examine viral infections

such as IAV. The resulting model is based on the isolation of primary human immune cells and subsequent purification of $CD14^+$ APCs. The purified $CD14^+$ APCs can be directly infected/stimulated, without the requirement of pre-priming of the immune responses [333,340,341]. The benefit of not pre-priming the cells is that immune responses are not skewed and mimic *in vivo* responses more closely [333]. A time-point of 24 hours was chosen to analyse as clinical symptoms and immune responses have been shown to manifest by then, which indicates very early immunosuppression by IAV infection [18,150,260,325,351,430]. Additionally, as asymptomatic carriage of pneumococcus is very common, especially in children, an early time point was of great interest [431,432]. Previously, the human *ex vivo* model was used to examine the effect of IAV infection on responses to *S.p*. using a lab-adapted virus and a clinical isolate of IAV along with in-house generated heat killed *S.p.* It demonstrated that IAV inhibited important pneumococcus driven innate cytokines secreted by human APCs and adaptive cytokines secreted by T cells [350]. Immune responses often differ when presented with distinct IAV strains and bacterial subtypes [302,309,351]. It was an aim of this project to confirm infectivity of H1N1 and H3N2 and to quantify the amount of NP produced from donor to donor. Additionally, another aim was to establish immune responses to heat killed *S.p.* using new batches of live H1N1 and a new clinical isolate of H3N2 to further characterise the human *ex vivo* model.

Here, we confirm that live H1N1 and H3N2 viruses have the ability to infect isolated $CD14^+$ APCs. To confirm this, an assay was designed which detected the presence and relative quantity of IAV NP using qPCR. Both H1N1 and H3N2 NP expression was amplified using qPCR. This served as a reliable indicator of infectivity as during IAV infection, NP is expressed intracellularly [340,352]. NP was detected in all IAV-infected cells. There was very little variability of H1N1 and H3N2 NP between donors, indicating that the H1N1 and H3N2 virus infectivity is very reproducible. H3N2 NP was less abundant than H1N1 NP; this may be because H1N1 is a lab-adapted virus, whereas H3N2 is a clinical isolate of IAV. These viruses were used throughout this study. This is a novel method for determining the relative quantification of IAV NP and it has been very effective in confirming how reproducible the IAV infections were.

We also provide further evidence in Chapter 2, that live IAV infection inhibits innate Th17 and Th1 polarising cytokines induced in response to *S.p.* infection. This was determined by analysing levels of secreted cytokines in supernatants from APCs using ELISA. To ensure effective characterisation of the new batches and clinical isolates of IAV and *S.p.*, a very high donor number was used for this study (n=20). This was to overcome any potential variabilities between donors as blood samples were from healthy human

donors. The cytokines analysed are involved in the Th17 and Th1 immune response to *S.p.*, along with anti-inflammatory cytokines which can inhibit Th17 and Th1 immune responses. Specifically, our findings have demonstrated that both H1N1 and H3N2 inhibited HKSP-induction of innate polarsing cytokines, IL-23, IL-6, and IL-27. IL-23 and IL-6 are essential in the Th17 response, whereas IL-27 has been implicated in both the Th17 and Th1 responses [182,228,246,354]. The inhibition of IL-27 is very interesting due to the apparent contradiction of its role in both the Th1 and Th17 response as it can both induce and inhibit these responses depending on which transcription factors are activated [182,228,354,376]. As HKSP-induction of IL-27 was itself inhibited by IAV, downward pressure on the Th17 responses cannot be attributed to it. H1N1 inhibited HKSP-induced IL-12p70, which is a Th1 cytokine, but H3N2 did not. IL-12p70 is involved in the production of IFN-γ, although there are conflicting reports on its effect on the clearance of *S.p*. [3,185,293,294]. It is surprising that H3N2 did not inhibit HKSP-induction of IL-12p70 as previously H3N2 was shown to inhibit these responses [350]. Commercially bought HKSP used for this study did not induce IL-12p70 very strongly, which may be why there was no inhibition of HKSP-induced IL-12p70 by H3N2. IAV did not inhibit pneumococcus-driven IL-1β, which is also essential in the Th17 response to *S.p*. [246]. Numerous other studies have also reported that IL-1β is induced by IAV in humans and mice [433–435], although induction occurred at later time points in these studies. Therefore, the lack of inhibition of IL-1β by IAV is unsurprising. There are numerous pathways whereby IL-1β is induced, which may be why IL-1β is not inhibited by IAV as with other Th17 cytokines [224]. We have also demonstrated that the inhibition of HKSP-induced IL-23, IL-6, IL-27, and IL-12p70 by IAV was not due to elevated levels of anti-inflammatory TGF-β and IL-10, or due to an increase in apoptosis. We also demonstrated that IAV infection inhibited Th17 and Th1 adaptive cytokines, IL-17A and IFN-γ. The adaptive response was studied by performing a MLR assay whereby infected/treated APCs from one donor are co-cultured with T cells from a different donor, resulting in an allogeneic response due to mis-matched MHC antigens. The inhibition of these cytokines was unsurprising as other studies have also demonstrated that IAV inhibits bacterial induction of IL-17A in mice [349,376]. Interestingly, HKSP-induction of IFN-γ was inhibited by both H1N1 and H3N2, and yet HKSP-induction of IL-12p70, which positively regulates IFN-γ was only inhibited by H1N1. Induction of IFN-γ can occur via two distinct pathways, which may explain this phenomenon [280–283]. As with innate responses, IAV-inhibition of HKSP-induced adaptive cytokines was not due anti-inflammatory TGF-β and IL-10. These studies are consistent with previous results showing that IAV inhibits innate responses that drive Th17 and Th1 polarisation, which

results in inhibition of adaptive Th17 and Th1 responses by human monocytes, and by APC-T cell co-cultures. This points to IAV having a more direct inhibitory effect on both innate and adaptive responses and raises the question of how the virus is inhibiting these responses.

As the inhibition of pneumococcus responses by IAV was shown to not be as a result of anti-inflammatory cytokines or an increase in apoptosis, other possible pathways where IAV could be both indirectly and directly targeting were explored. As a result, another aim of this project was to determine the levels of type I IFN present across treatments as various studies have implicated induction of type I IFN as being responsible for inhibition of pneumococcus responses by IAV [2,3,436]. In Chapter 3, we have presented novel findings demonstrating that IAV mediated inhibition of Th17 responses were not due to type I IFNs in human APCs. Expression of IFN-α mRNA was only amplified in 1 out of 4 donors, except for those treated with the positive control, Poly(I:C). Levels of IFN-α mRNA were higher in cells treated with HKSP alone than in cells co-treated with both HKSP and IAV. Very low levels of IFN-β mRNA was detected in all samples, except for cells treated with Poly(I:C), which induced very high levels of IFN-β. There was no significant increase in levels of IFN-β mRNA in HKSP and IAV co-treated cells compared HKSP-alone treated cells. The inhibition cannot be attributed to type I IFN mRNA expression as minor amplification of IFN-α occurred in only one donor and any amplification which did occur was extremely low, and IFN-β was not amplified beyond basal levels. The inhibition cannot be due to IFN-α protein production as although IFN-α was detected in cells co-treated with HKSP and H1N1, no IFN-α was detected in cells co-treated with HKSP and H3N2, where inhibition was also observed. Additionally, IFN-β protein production cannot be the reason for the inhibition as the levels of IFN-β protein in co-infected cells was at most at basal levels such as those measured in untreated cells, where no inhibition of immune responses occurs. This work corroborates other research suggesting that IFN-α is not produced until 3 days post-influenza infection [2], which is later than the time point we have chosen to study. A reason for late production of type I IFNs can be attributed to the influenza protein, NS1, which blocks IRF3, IRF7, and NF-κB, resulting in the inhibition of type I IFNs [437]. Additionally basal levels of IFN-β protein has been shown to be insufficient to inhibit Th17 responses in humans [412]. This is a novel finding which shows that production of type I IFNs are not involved in the inhibition of pneumococcus responses in human monocytes. This is the first study to demonstrate that inhibition of Th17 responses by IAV occurs without Type I IFN production.

As IAV inhibition of HKSP responses could not be indirectly attributed to the presence of type I IFN, it suggested that IAV may be having more of a direct effect on immune responses to HKSP. Therefore, the immune response to *S.p.* was researched to find possible areas where IAV may target. TLR recognition is a fundamental part of the immune responses to *S.p.* The specific TLRs involved in *S.p.* recognition are TLR2, TLR4, and TLR9 [96]. In mice, research has shown that in the absence of TLR2 signalling, *S.p.* infection occurred more efficiently during influenza co-infections [316]. TLR2 has been shown to recognise pneumococcal cell wall components including LTA [123]. TLR4 is triggered in responses to *S.p.* by recognising PLY in humans and mice [126–128], but is also triggered by LPS from Gram-negative bacteria [438]. TLR9 recognises pneumococcal DNA comprising unmethylated CpG motifs [126–128,130]. In Chapter 3, a large focus of the research involved determining if IAV was directly inhibiting *S.p.* associated TLR induction. The findings detailed in Chapter 3, demonstrate that each of the *S.p.* associated TLRs are targeted directly by IAV infection. IAV appears to be exerting a stronger inhibitory effect on the TLR2 and TLR9 pathways than the TLR4 pathway. The differential effects of IAV between the TLRs may be explained by slight variations in the signalling pathways of these receptors. For instance, the TLR2 and TLR9 pathways are both MyD88-dependent, meaning they require recruitment of the adaptor molecule, MyD88 in order to trigger downstream signalling cascades [133–135,439]. However, the TLR4 pathway is MyD88-independent, meaning it can recruit MyD88, or it can recruit a different adaptor molecule TRIF, both of which trigger downstream signalling [134,135]. As TLR4 can signal through two distinct pathways, this may explain why TLR4 agonism appears to be less susceptible to inhibition by IAV that TLR2 and TLR9 agonism. The more robust nature of the TLR4 pathway may be why one study suggested that a TLR4 agonist encouraged innate immunity against *S.p.* during co-infection with IAV [440]. Induction of IL-23 in response to TLR2 and TLR9 agonism was inhibited by both strains of IAV, which is interesting as in Chapter 2 HKSP-induction of IL-23 was also inhibited by IAV. Induction of TGF-β by each of the TLRs was inhibited by both H1N1 and H3N2. It should be noted that although TLR-induction of TGF-β was inhibited by IAV, HKSP-induction of TGF-β (Chapter 2) was not. As mentioned previously, this may be due to the agonists being mono-agonists. As whole HKSP is detected by multiple TLRs, it is likely that immune responses are less vulnerable to inhibition compared to when a single TLR is being stimulated. TLR-induced IL-1β, IL-6, IL-27, and IL-12p70 were not affected by IAV infection, suggesting that IAV is having a specific inhibitory effect on TLR-induced IL-23 and TGF-β. As IL-23 is a very important

cytokine involved in Th17 commitment, this of significant interest, and merited further investigation.

IL-23 expression is induced by RORC, which is a transcription factor specific to the Th17 response [187,190–193,196–201]. With a view to providing additional mechanistic insight into how IAV inhibits *S.p.* and TLR agonist induction of IL-23, we sought to determine if IAV was directly targeting TLR-induction of RORC. Results presented in Chapter 3, indicate that TLR2 agonist induction of RORC was not inhibited by IAV, although TLR9 agonist induction of RORC was inhibited by both H1N1 and H3N2. TLR2 agonist induction of RORC was inconsistent in our model, with only one donor out of three inducing RORC above basal levels. A study performed using human B cells found that TLR2 agonism did not induce RORC, and found that TLR9 agonism did induce RORC, which corroborates the data presented here [441]. Although, this study did not hypothesise why this may have occurred, as mentioned previously, it may be due that RORC is induced by TLR2 agonism at a different time point as RORC has been shown to be induced at different time points [206]. The inhibition of RORC by IAV may be playing a pivotal role in the downstream inhibition of cytokines involved in the Th17 response. This effect of IAV on TLR-induction of RORC has not been previously demonstrated in human APCs and may be of use when developing therapeutic targets. For instance, if RORC expression was restored, this may prevent the downstream inhibition of cytokines. A study researching the role of RORC on psoriasis, demonstrated that Imiquimod, a topical prescription medicine, upregulated RORC at both the mRNA and protein level [442], therefore similar treatments (although not topical) may be beneficial to trial for treatment of respiratory bacterial infections.

As *S.p.*-associated TLR pathways were each inhibited by IAV, we sought to investigate other TLRs. Multiple studies on TLR5 signalling demonstrated that it may induce protective responses during bacterial infections [382–384]. Additionally, TLR5 agonism has elicited protection against *Clostridium difficile*, vancomycin-resistant *Enterococcus*, and *S.p.* in mice [385–387]. This is interesting as TLR5 is induced by flagellin which is not a component of *S.p.* Due to this, we sought to establish whether IAV also inhibited TLR5 agonism in APCs. In this study, we have established that IAV infection does not inhibit TLR5-induction of Th17 and Th1 polarising cytokines. Other research using mouse models have demonstrated that TLR5 agonsim can exert therapeutic benefits when combined with antibiotic administration during treatment of IAV and *S.p.* co-infections [388]. Therefore, an aim of this study was to determine whether a TLR5 agonist can be used to restore IAV inhibited immune responses to *S.p.* in human APCs.

In Chapter 3, we have established that TLR5 agonism restored levels of HKSP-induced IL-23 and IL-27 which were previously inhibited by IAV. Additionally, in cells co-treated with HKSP, IAV, and a TLR5 agonist, levels of IL-12p70 were induced beyond those in cells treated with HKSP alone. These results present a novel mechanism for restoring Th17 and Th1 polarising cytokines in human APCs, which may also prevent inhibition of Th17 and Th1 adaptive cytokines. As discussed before, there has been research performed in this area previously, although in mouse models. As immune responses in mice and humans are not always conserved, it is of great import that research is also carried out in human models. These results corroborate those generated in mouse models, which may help to demonstrate the possibility of utilising TLR5 agonists to boost immune responses. Such treatments may prove to be indispensable especially when considering the great task of overcoming AMR. Boosting immune responses via TLR agonists may prove to be a very effective tactic for not only improving the efficacy of antibiotics (which will be less and less effective by themselves as time passes), but may even be sufficient alone. In order to provide balance, the use of such treatment strategies must also be cautioned due to the known pathogenic effects that elevated Th17 responses can exert [198,295–297]. Although, other studies in mice observed that lung tissue fully recovered 1 week after administration despite very high inflammatory responses before, which points to the effects of flagellin being somewhat “self-limiting” [387]. This may prove to be key in developing future anti-microbial treatments, but as with other research, this needs to be verified in human models. It should also be noted that studies which analysed the effect of TLR5 signalling using knock-down mice ($TLR5^{-/-}$), were performed using Gram-negative bacteria, and these studies found that TLR5 signalling conferred protection against bacterial infections [382,383]. However, all studies which utilised a TLR5 agonist as a treatment against bacterial infection were performed using Gram-positive bacteria [385–387]. With the exception of studies using *S.p.,* all other bacteria were flagellated [382–387]. Although *S.p.* do not contain flagella and have not traditionally been associated with TLR5, it has been hypothesised that a reason why TLR5 is beneficial to *S.p.* clearance is because TLR5 signalling also induces a MyD88-dependent signalling cascade, which as mentioned before is a key pathway triggered by *S.p.* infection [387]. This creates the question of how IAV inhibits TLR2- and TLR9- recruitment of MyD88, but does not appear to affect TLR5 recruitment of MyD88. The MyD88-dependent pathway is identical regardless of which TLR triggers it. As TLR5 agonism triggers Th17 responses even in the presence of IAV infections, this may point to TLR5 utilising a distinct pathway of which there is no knowledge of. These findings may not only be significant for IAV-bacterial infections, but may also be of interest for

those studying the field of autoimmunity as the more that is known about the Th17 pathway, the easier it may be to develop better treatment strategies. This may prove to be a very important finding and merits further research.

To elucidate how IAV infection inhibits immune responses, the components of IAV were examined. The surface glycoproteins HA and NA are components of IAV, which act as viral antigens [33]. The HA protein is of particular interest as it serves as a target for influenza vaccine development, which is the primary approach utilised in the prevention and regulation of influenza infections [6,7]. Due to the importance of HA and as live IAV has been shown to inhibit both immune responses to HKSP and TLR agonism (summarised in Chapter 2 and Chapter 3), the effect of HA on both HKSP and TLR agonism was examined, with a view to determining if HA may be partially responsible for IAV mediated immune inhibition (summarised in Chapter 4). In this study, HKSP-induced IL-27 and IL-12p70 were inhibited by both H1N1 and H3N2 HA. It is interesting that HA is having an impact on these particular cytokines as IL-12p70 is a Th1 polarising cytokine and although IL-27 has a role in Th17 differentiation, it also contributes to Th1 immunity [354,423]. HA did not inhibit HKSP-induction of Th17 polarising cytokines (IL-23, IL-6, or IL-1β), therefore it is possible that HA has a particular effect on Th1 polarising cytokines. It may be that different viral components target distinct Th cells, therefore it may be of interest to examine other viral components to determine their effects on the same cytokines. After analysing innate responses, the effect of HA on adaptive responses was explored. HKSP-induced IL-17A and IFN-γ were both inhibited by H1N1 HA and H3N2 HA. It is curious that HKSP-induction of IL-17A was inhibited by HA and yet HKSP-induction of IL-23 was unaffected by HA as IL-23 is known to be important in the expansion of Th17 cells, which produce IL-17A. A study in mice found that IL-17A can be induced by IL-6 and TGF-β, in the absence of IL-23 [246]. However, neither HKSP-induced IL-6 or TGF-β were inhibited by HA. This points to HA having a specific inhibitory effect on IL-17A, without affecting the innate responses which inform adaptive responses. Although, the results obtained using HA are of interest, it seems unlikely that HA is the main cause of inhibition by IAV as HA does not inhibit HKSP-induction of any Th17 polarising cytokines. Different viral components such as NA or NS1 may be exerting more profound inhibitory effects. Or perhaps it is a combination of each viral component working in synergy that results in IAV mediated inhibition of immune responses. The effect of HA should not be minimised though, especially as it is a common component of influenza vaccines [6,7]. Pneumococcal vaccines are often given concurrently with influenza vaccines (albeit at a different site) [108]. Keeping this in mind

and considering that HA has inhibited HKSP responses, it would be recommended to continue to administer vaccines at different sites, but also to perhaps stagger administration of these vaccines. This may improve efficacy of the pneumococcal vaccine, and eliminate potential interference from HA in the influenza vaccine.

An aim of this study was to continue the research examining *S.p.*-associated TLR agonists (summarised in Chapter 3). To enable this, the effect of HA (which inhibited HKSP responses) on TLR agonism was examined in our human *ex vivo* model. The further research performed in this chapter, demonstrated that H1N1 HA and H3N2 HA inhibited TLR2-induced TGF-β. As mentioned before, it is interesting that TLR-induced TGF-β was inhibited by both HA and live IAV, yet HKSP-induced TGF-β was not inhibited by either. This is most likely due to the mono-agonist nature of the TLR2 agonist which exclusively activates the TLR2 receptor compared with whole HKSP which activates multiple receptors, and is therefore less vulnerable to inhibition. TLR9-induction of IL-23 was inhibited by H1N1 HA, but not H3N2 HA. As mentioned before, the inhibitory effect of IAV on TLR9-induction of IL-23 was also very strong. Even though HA inhibited TLR9-induction of IL-23, it is unlikely that IAV-mediated inhibition is due to this entirely as only H1N1 HA inhibited TLR9-induced IL-23, yet live H3N2 also inhibited TLR9 agonism. TLR9-induction of TGF-β was not inhibited by HA, but was inhibited by live IAV. Additionally, HA did not inhibit HKSP-induced IL-23, therefore there are additional aspects and mechanisms to consider.

Current clinical trials are ongoing to determine the safety and efficacy of using a TLR5 agonist as an adjuvant to the influenza vaccine [420–422]. As HA is also a common component of influenza vaccines, any possible interactions between HA and flagellin should be explored. It was established that HA treatment did not inhibit TLR5-induction of Th17 and Th1 polarising cytokines. This is in keeping with studies in Chapter 3, where live IAV was shown to not have an inhibitory effect on TLR5 agonism. This apparent lack of any inhibitory effect by HA on TLR5 agonism is reassuring, especially as the TLR5-adjuvant influenza vaccine currently being trialled may be able to overcome the issues encountered with elderly people, whereby they require a higher dose of HA to induce immunity [26,420].

As a TLR5-adjuvant influenza vaccine may used commonly in the future, the combined effect of HA, a TLR5 agonist, and HKSP was examined. These were examined together as the pneumococcal vaccine is commonly administered simultaneously with the influenza vaccines [108]. TLR5 agonism also restored HKSP-induced IL-27 and IL-12p70 which had been inhibited by HA treatment, and additionally boosted levels of IL-

23 and IL-1β to above those induced by HKSP alone treated cells. These results using HA treatment, mirror those obtained using IAV. This is the first study to analyse the effect of H1N1 and H3N2 HA on TLR agonism in human APCs. As flagellin (TLR5 agonist) is very effective at boosting Th17 polarising cytokines, individuals with autoimmune conditions may not eligible for administration with this potential new adjuvant influenza vaccine, due to the detrimental role of Th17 cytokines in autoimmune conditions.

Overall, this project has demonstrated the use of our human *ex vivo* model and has highlighted the need to also include human models in the field of research. It is well known that IAV predisposes individuals to *S.p.* infections, however the many contradictions surrounding the possible reasons behind this can be difficult to navigate. The dogma for a number of years has been that the inhibition of *S.p.* responses is due to type I IFNs, however these responses are known to be inhibited by viral NS1 [270–272]. We have shown that type I IFNs are not produced, yet inhibition of *S.p.*-induced immune responses by IAV occurs regardless. This pointed to IAV directly having an effect on immune responses, which directed our focus towards possible mechanisms of interest. We identified that IAV targets specific *S.p.*-associated TLRs and does not target TLR5, which has not been linked with detection of *S.p.* TLR5 agonist treatment reversed inhibition of *S.p.* responses by IAV and shows great promise for improving outcomes of bacterial infections. The use of TLR agonists as possible therapeutic treatments is an exciting and innovative area of research of which there is still much to explore. The areas of research where TLR agonism is applicable are manifold including vaccine adjuvants, cancer therapies, and allergy treatments [443–447].

Many of the studies found in the literature have been performed in mouse models, therefore this is an area of great novelty for any studies where a human model is utilised. As mentioned previously there should always be caution exerted when using treatments which alter immune responses as the knock-on effects can be detrimental. Th17 cells, although very effective in the clearance of bacterial infections, can elicit highly pathogenic effects when produced in abundance. For instance, IL-17 (which is produced by Th17 cells) has been shown to be responsible, for autoimmune diseases such as psoriasis, Crohn's disease, rheumatoid arthritis, MS, and asthma [198,295–297]. Encouragingly, the effects of TLR5 agonism, specifically flagellin, have been shown to be transient and "self-limiting" as lung tissue in mice fully recovered from high inflammatory responses 1 week after administration [387]. However, another potential problem to consider is the relapsing nature of such autoimmune issues. Therefore, longer-term studies should be performed to help determine how likely such a treatment

may be to cause pro-inflammatory autoimmune conditions. If such issues are rectified (or indeed shown not to be problematic), TLR treatments may become invaluable in aiding the treatment of bacterial infections, which is of ever-growing necessity due to the increase in anti-microbial resistance.

5.1 NOVEL FINDINGS OF THIS STUDY

1. Demonstrated continued and consistent inhibition of *S.p.*-induced human (innate and adaptive) Th17 and Th1 responses by new batches of H1N1 and new clinical isolates of H3N2
2. Determined that early inhibition of Th17 responses by IAV is not due to the presence of type I IFNs in human APCs
3. Established that IAV infection directly inhibits *S.p.* associated TLRs in human APCs
4. Ascertained that TLR9-induction (but not TLR2-induction) of RORC is inhibited by IAV in human APCs, which may be key in downstream inhibition of cytokines
5. Verification that IAV does not target TLR5 agonism in human APCs
6. Demonstrated a restoration of *S.p.*-induced Th17 and Th1 responses during IAV infection by using a TLR5 mono-agonist as a treatment in human APCs
7. Established a link between TLR5 signalling and Th17 responses, which may provide novel mechanistic insights into use of TLR5 agonism in bacterial infections, particularly those caused by Gram-positive bacteria
8. Identified that HA is not solely responsible for IAV-mediated inhibition of TLR responses, thus providing rationale to explore other IAV viral components

5.2 FUTURE WORK

There are many interesting avenues with which to continue this important research. A laboratory-strain of H1N1 was used for this study as this strain is very widely used and it facilitated comparing our findings to other studies on mice and human models. However, it may be of interest to also supplement future work using a clinical isolate of H1N1 along with additional strains of H3N2 and other subtypes. This is with a view to eventually using our human *ex vivo* model to help identify which strains of IAV are most likely to predispose individuals to secondary bacterial infection. The ideal scenario would involve aligning the Viral Immunology Group at DCU with an influenza centre, where we would be given IAV strains currently in circulation. Using these strains, we could use the model to determine the effect of these strains on Th17 responses. The results obtained

in our laboratory could be compared to the percentage of secondary infections in the population to determine how suitable this method is in predicting the likelihood of individual strains causing secondary bacterial infections. Ultimately, this method may be used to help advise, not only vaccination strategies, but also the clinical management of infections. For instance, if a circulating influenza strains is known to be particularly immunosuppressive, prophylactic treatments may be recommended for very vulnerable individuals.

In the more immediate future, it would be interesting to study the effects of the influenza surface glycoprotein, NA. It has been suggested that influenza vaccine development has underexploited NA immunity, however there are now numerous studies which have demonstrated the potential benefits of including NA in vaccine formulations [448–452]. The supposed underuse of NA as an antigen in vaccines was due to many issues including NA being unstable which resulted in conflicting reports of NA immunogenicity [453]. However, as NA is now being considered as a component for broad-spectrum influenza vaccines, the effects of NA on responses to *S.p.* and TLR agonism may be an important addition to the field [448,450,454].

Additionally, the influenza viral protein, NS1 may also be of interest. NS1 is known to inhibit immune host antiviral responses, such as the production of type I IFNs [270–272]. The antagonism of host immune responses by NS1 differs between IAV strains, but NS1 has been shown to inhibit IRF3 and NF-κB transcription [171,172,455]. Due to this, NS1 has been identified as a possible target for therapies to treat IAV infections. A chemical inhibitor of NS1 (JJ3297) has been effective in preventing influenza viral replication [456]. As very low levels of type I IFN was detected in cells in our human *ex vivo* model, it may be attributed to NS1. Therefore, it may be of interest to try and determine what else NS1 may be targeting. The effect of NS1 treatment on *S.p.* and TLR agonism would also be beneficial to give more insight into the mechanisms behind IAV-mediated inhibition of *S.p.* responses. Additionally, the effect of using multiple agonists simultaneously should be explored as a study has shown that ligands with specific affinity for TLR2/6 and TLR9 have been shown to induce protection when combined against influenza and also in the early stages of *S.p.* infection in mice [130,403].

To complement results examining expression of RORC, which is induced by STAT3, it may be insightful to study the levels of phosphorylation of both STAT1 and STAT3. These are transcription factors which heavily influence whether Th17 responses are induced or inhibited. For example, STAT1 has a profound inhibitory effect on Th17 responses but induces Th1 responses, whereas STAT3 strongly induces Th17 responses and inhibits Th1 responses [203,233–236]. By determining the levels of these transcription factors, it may give insight as to whether Th1 and Th17 responses are

affected differentially across treatments. It may also be beneficial to study additional time-points with a view to expanding the knowledge on the capability of TLR2 to induce RORC.

To further the research already completed using the TLR agonists, the effect of IAV and components of IAV on the adaptive immune responses to TLR agonism would be an important addition and add to the knowledge base, especially regarding using TLR5 agonism as a treatment strategy.

This research is not only very promising for creating a better understanding of how IAV inhibits *S.p.* infections but also may be helpful to those researching treatments for pro-inflammatory autoimmune conditions. It may be useful to study whether viral components could be utilised to reduce inflammation in patients with these conditions, to alleviate symptoms, both short-term and long-term.

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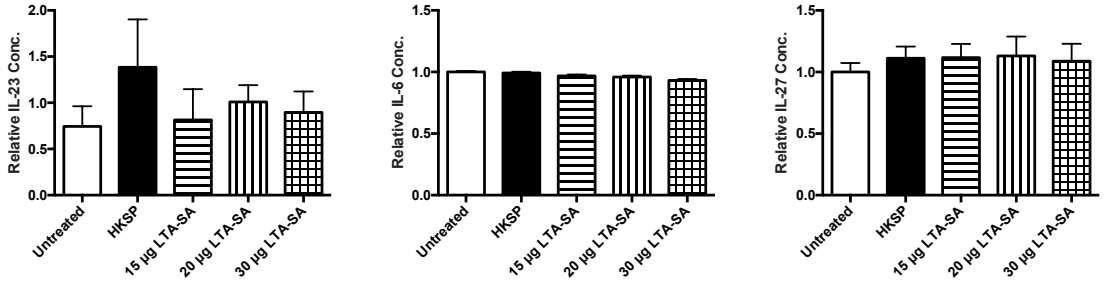
APPENDIX 1 – CHAPTER 3

APPENDIX 1.1: THE OPTIMAL DOSE OF TLR2 AGONIST

To determine the optimum dose of the TLR2 agonist, dose responses were performed. Lipoteichoic acid from *Staphylococcus aureus* (LTA-SA) (Invivogen) was used as a TLR2 agonist at a range of doses from 15 μg to 30 μg. The TLR2 agonist, LTA-SA induces pneumococcus-driven cytokines most consistently at a concentration of 20 μg. The 20 μg dose was the strongest IL-23 inducer (n=3). Induction of IL-6 did not occur above baseline levels, regardless of treatment (n=3). The 20 μg dose of LTA-SA induced a slightly stronger IL-27 response than the 30 μg dose (n=3). Additionally, the 20 μg dose of LTA-SA induced a stronger induction of IL-12p70 than others, however as most values were below limit of detection, therefore data could not be normalised to reflect multiple donors (data not shown).

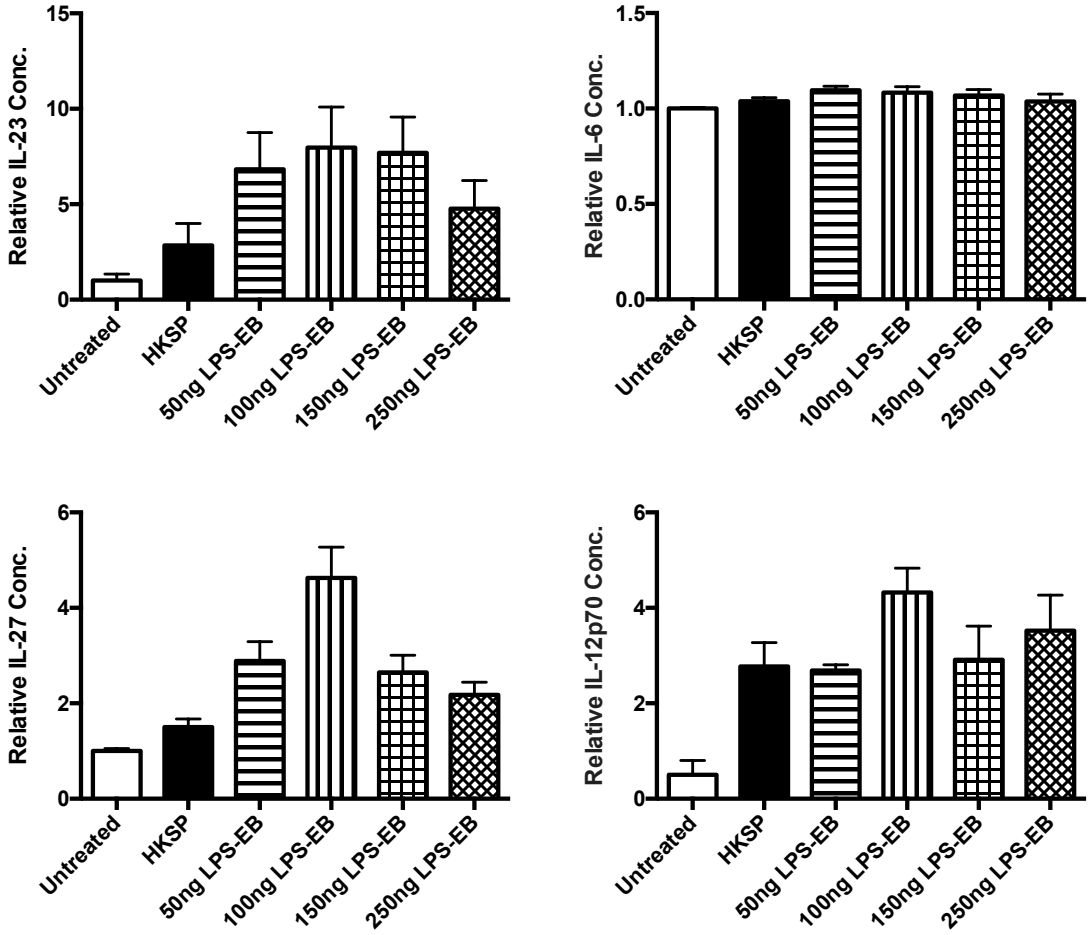


Appendix 1.1 TLR2 agonist (LTA-SA) induces pneumococcus-driven cytokines most consistently at a concentration of 20 μg. The levels of IL-23, IL-6, and IL-27 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, LTA-SA at doses of 15 μg, 20 μg, and 30 μg or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 experimental repeats of each treatment in the same donor (n=3).

APPENDIX 1.2: THE OPTIMAL DOSE OF TLR4 AGONIST

To determine the optimum dose of the TLR4 agonist, dose responses were performed. Ultra-pure Lipopolysaccharide from *E. coli* (LPS-EB) (Invivogen) was used a TLR4 agonist at a range of doses from 50 ng to 250 ng.

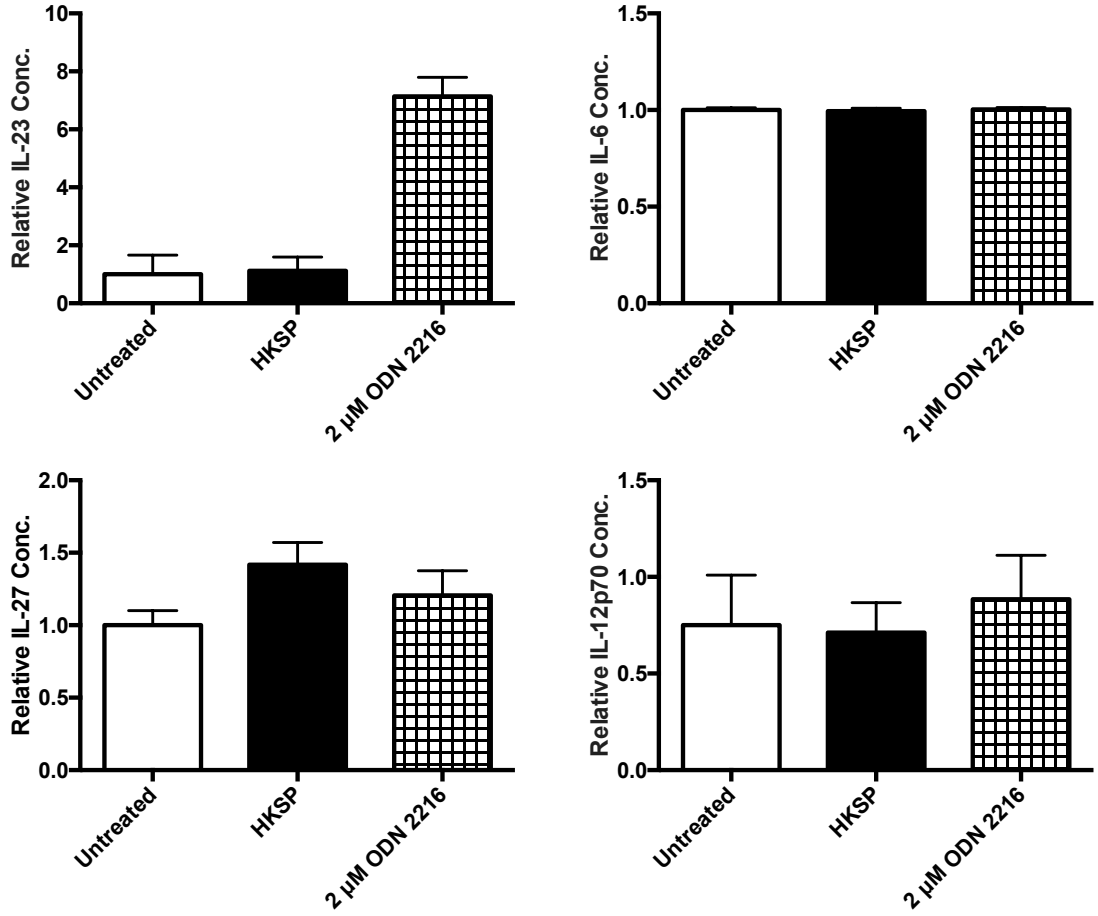
The TLR4 agonist, LPS-EB induces pneumococcus-driven cytokines most consistently at a concentration of 100 ng. The 100 ng dose induced the strongest responses of IL-23, IL-27, and IL-12p70 (n=3). As with the TLR2 agonist, induction of IL-6 did not occur above baseline levels, regardless of TLR4 treatment (n=3).



Appendix 1.2 TLR4 agonist (LPS-EB) induces pneumococcus-driven cytokines most consistently at a concentration of 100 ng. The levels of IL-23, IL-6, IL-27, and IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, LPS-EB at doses of 50 ng, 100 ng, 150 ng, and 250 ng or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 experimental repeats of each treatment in the same donor (n=3).

APPENDIX 1.3: THE OPTIMAL DOSE OF TLR9 AGONIST

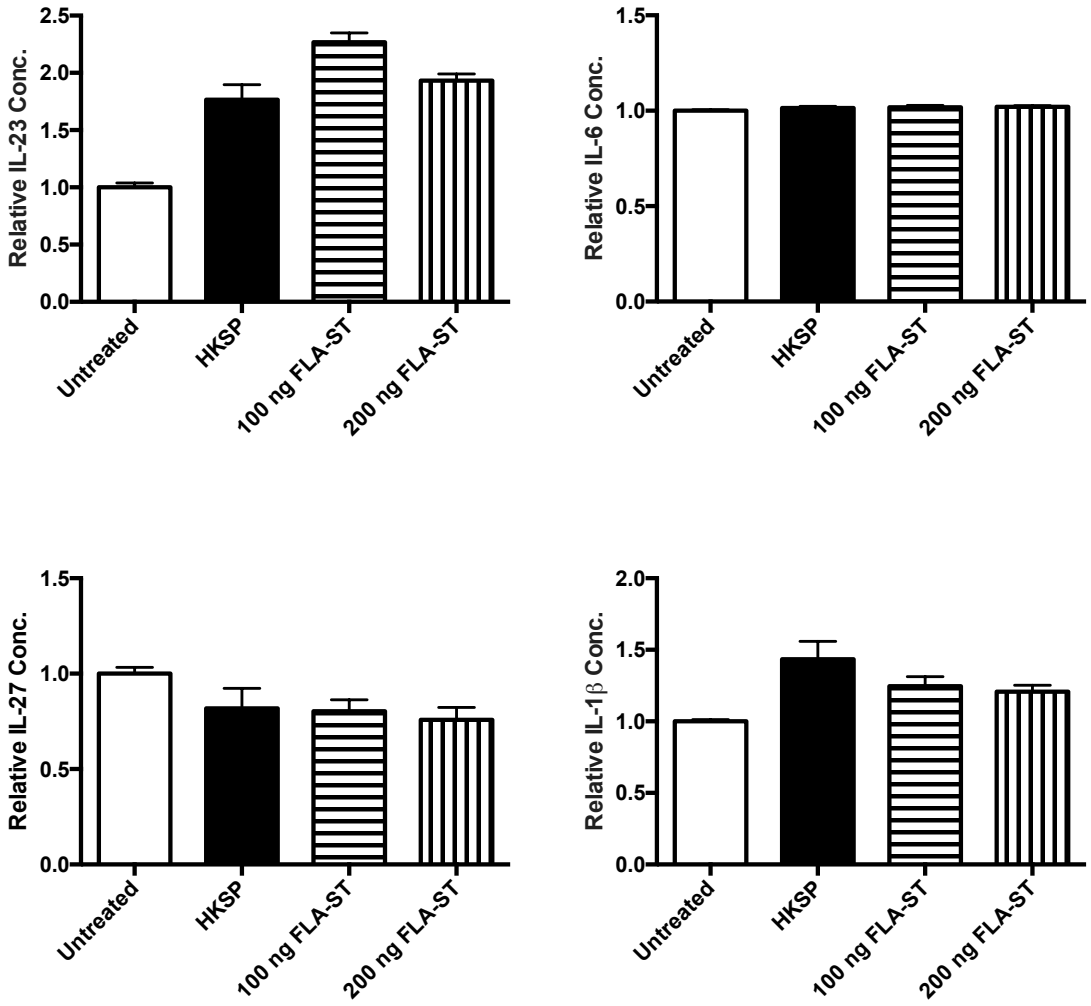
To determine if the TLR9 agonist could induce pneumococcus-driven cytokines were stimulated with 2 μM of the chosen TLR9 agonist, Class A CpG oligonucleotide (ODN 2216) (Invivogen). The TLR9 agonist, ODN 2216 induces pneumococcus-associated cytokines at a concentration of 2 μM. The 2 μM dose induced very robust levels of IL-23 (n=3). As with previous TLR agonists, IL-6 was not induced past baseline levels by the TLR9 agonist (n=3). The TLR9 agonist induced both IL-27 and IL-12p70 above baseline levels (n=3).



Appendix 1.3 TLR9 agonist (ODN 2216) induces pneumococcus-driven cytokines at a concentration of 2 μM. The levels of IL-23, IL-6, IL-27, and IL-12p70 secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, ODN 2216 at a dose of 2 μM or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 experimental repeats of each treatment in the same donor (n=3).

APPENDIX 1.4: THE OPTIMAL DOSE OF TLR5 AGONIST

To determine the optimum dose of the TLR5 agonist, dose responses were performed. Flagellin from *Salmonella typhimurium* (FLA-ST) (Invivogen) was used a TLR5 agonist at 100 ng and 200 ng. The TLR5 agonist, FLA-ST induces pneumococcus-driven cytokines most consistently at a concentration of 100 ng. The 100 ng dose induced the strongest responses of IL-23, IL-27, and IL-1β (n=3). Induction of IL-6 did not occur above baseline levels, regardless of TLR5 treatment (n=3).



Appendix 1.4 TLR5 agonist (FLA-ST) induces pneumococcus-driven cytokines most consistently at a concentration of 100 ng. The levels of IL-23, IL-6, IL-27, and IL-1β secreted by $CD14^+$ APCs following 24 hr treatment with HKSP, LFLA-ST at doses of 100 ng and 200 ng or untreated as a control were determined by ELISA. Each column represents normalised mean cytokine levels + SEM of 3 experimental repeats of each treatment in the same donor (n=3).

APPENDIX 2 – CHAPTER 4

APPENDIX 2.1: NEW BATCHES OF HAEMAGGLUTININ DISPLAY SIMILAR EFFECTS TO PREVIOUS BATCHES

To facilitate further studies on the effect of HA treatment on TLR agonism, it was important that new batches of HA were characterised fully. The effect of new HA batches (received from NIBSC) on the response to pneumococcus was analysed to add to important work already completed. The level of inhibition of HKSP responses by HA was compared between different batches by cross-checking differences in fold-change cytokine expression between HKSP-treated cells and HKSP and HA co-treated cells. The results obtained show that the new batches of HA do not inhibit HKSP-induction of IL-23, no inhibition occurred with previous HA either. No inhibition of HKSP-induced IL-6 occurred in response to treatment with new HA batches, however previously HKSP-induction of IL-6 was inhibited by the 1 μg dose of H1N1 HA. All treatments with new HA inhibited HKSP-induction of IL-27, whereas previously, only the H1N1 HA inhibited HKSP-induction of IL-27. No inhibition of HKSP-induced IL-1β occurred despite HA treatment; this occurred with both old and new batches of HA. Using new batches of HA, the 1 μg dose of both H1N1 HA and H3N2 HA inhibited HKSP-induction of IL-12p70. Previously, the 3 μg dose of H1N1 HA also inhibited HKSP-induction, although a reduction was observed using new batches, this was no statistically significant. The 3 μg dose of H3N2 HA (new batch) inhibited HKSP-induced IL-10, whereas no inhibition occurred with previous batches of HA. No inhibition of HKSP-induced TGF-β occurred in response to new batches of HA, whereas previous batches of H1N1 HA did inhibit HKSP-induced TGF-β.

Appendix Table 2.1 Comparison between inhibition of previous HA batch results against current results generated with new batches of HA

Cytokine	H1N1 HA				H3N2 HA			
	Previous*		New**		Previous*		New**	
	1 μg	3 μg	1 μg	3 μg	1 μg	3 μg	1 μg	3 μg
IL-23	-3.4	-7.42	0.11	0.12	-2.22	-2.6	-0.04	0.06
IL-6	0.33	0.27	0.02	0.01	-0.28	-0.05	0.01	0.05
IL-27	0.29	0.55	0.17	0.14	0.04	0.2	0.18	0.27
IL-1β	-0.29	-0.32	0.05	0.07	-0.09	-0.2	-0.01	0.26
IL-12p70	0.8	0.78	0.25	0.21	-0.01	0.4	0.25	0.13
IL-10	-0.22	-0.08	-0.02	0.02	-0.37	-0.37	0.02	0.08
TGF-β	0.34	0.28	0.07	0.11	0.03	0.02	0.11	0.17

Yellow indicates inhibition of HKSP by HA, whereas blue indicates no inhibition. Numerical values represent the difference between HKSP relative concentrations and HKSP and HA relative concentrations. Negative numbers indicate that co-treated cells induced higher amounts of cytokine than HKSP alone treated cells. *n=4; **n=3

APPENDIX 2.2: NEW BATCHES OF HAEMAGGLUTININ HAVE SIMILAR IMMUNOSUPPRESSIVE EFFECTS ON ADAPTIVE RESPONSES TO PREVIOUS BATCHES

Upon receipt of new HA batches from the NIBSC, previous studies were continued to determine if any differences occurred, with a view to furthering future research using the HA samples. The level of inhibition of HKSP responses by HA was compared between different batches by cross-checking differences in fold-change cytokine expression between HKSP-treated cells and HKSP and HA co-treated cells. The results using new batches of HA obtained for IL-17A, IFN-γ, and TGF-β were in keeping with what was previously obtained. HKSP-induced IL-17A was inhibited by new batches of both H1N1 and H3N2 HA at the 3 μg dose. However, only the 3 μg dose of H3N2 HA had previously inhibited HKSP-induced IL-17A. All HA treatments (using both old and new batches of HA) inhibited HKSP-induction of IFN-γ. No inhibition of HKSP-induced TGF-β despite HA treatment; this occurred using both old and new batches of HA. Elevation of IL-10 did not occur using both old and new HA, indeed new batches of HA actually inhibited HKSP-induction of IL-10.

Appendix Table 2.2 Comparison between inhibition of previous results against current results generated with new batches of HA

Cytokine	H1N1				H3N2			
	Previous*		New**		Previous*		New**	
	1 μg	3 μg	1 μg	3 μg	1 μg	3 μg	1 μg	3 μg
IL-17A	0.29	0.2	0.21	0.3	0.33	0.36	-0.05	0.34
IFN-γ	0.44	0.59	0.99	0.96	0.27	0.34	0.61	0.86
TGF-β	-0.14	-0.28	0.03	-0.05	-0.55	-0.12	-0.07	-0.03
IL-10	-0.14	-0.27	0.17	0.46	-0.55	-0.11	0.26	0.38

Yellow indicates inhibition of HKSP by HA, whereas blue indicates no inhibition. Numerical values represent the difference between HKSP relative concentrations and HKSP and HA relative concentrations. Negative numbers indicate that co-treated cells induced higher amounts of cytokine than HKSP alone treated cells. *n=3; **n=3.